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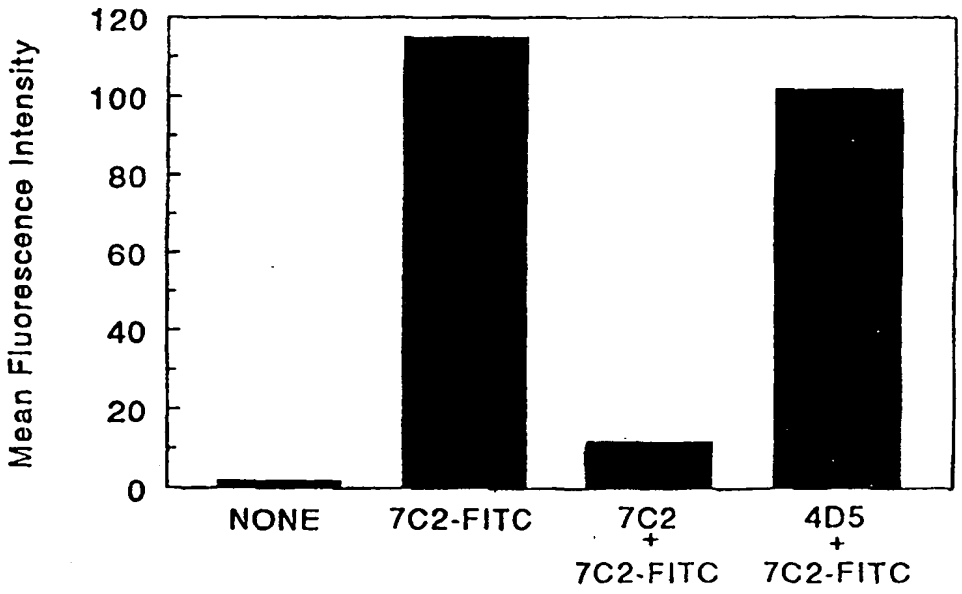



(57) Abstract

Anti-ErbB2 antibodies are described which bind to an epitope in Domain 1 of ErbB2 and induce cell death via apoptosis. Various uses for these antibodies are also described.

# ANTI-ErbB2 ANTIBODIES

## BACKGROUND OF THE INVENTION

### Field of the Invention

This invention relates generally to antibodies which bind the ErbB2 receptor. In particular, it pertains to anti-ErbB2 antibodies which bind to an epitope in Domain I of ErbB2 and induce cell death via apoptosis.

### Description of Related Art

Transduction of signals that regulate cell growth and differentiation is modulated in part by phosphorylation of various cellular proteins. Protein tyrosine kinases are enzymes that are involved in this process. Receptor protein tyrosine kinases are believed to direct cellular growth via ligand-stimulated tyrosine phosphorylation of intracellular substrates. The class I subfamily of growth factor receptor protein tyrosine kinases includes the 170 kDa epidermal growth factor receptor (EGFR) encoded by the *erb*B1 gene. *erb*B1 has been causally implicated in human malignancy. In particular, increased expression of this gene has been observed in carcinomas of the breast, bladder, lung, head, neck and stomach. Monoclonal antibodies directed against the EGFR have been evaluated as therapeutic agents in the treatment of such malignancies. For a review, see Baselga *et al. Pharmac. Ther.* 64:127-154 (1994). See also Masui *et al. Cancer Research* 44:1002-1007 (1984).

Wu *et al. J. Clin. Invest.* 95:1897-1905 (1995) recently report that the anti-EGFR monoclonal antibody (mAb) 225 (which competitively inhibits EGF binding and blocks activation of this receptor) could induce the human colorectal carcinoma cell line DiFi (which expresses high levels of EGFR) to undergo $G_1$ cell cycle arrest and programmed cell death (apoptosis). Addition of IGF-1 or high concentrations of insulin could delay apoptosis induced by mAb 225, whereas $G_1$ arrest could not be reversed by addition of IGF-1 or insulin.

The second member of the class I subfamily, $p185^{neu}$, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The activated form of the *neu* protooncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of *neu* (called *erb*B2 or HER2) is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon *et al.*, *Science*, 235:177-182 (1987); and Slamon *et al.*, *Science*, 244:707-712 (1989)). Accordingly, Slamon *et al.* in US Pat No. 4,968,603 describe various diagnostic assays for determining *erb*B2 gene amplification or expression in tumor cells. To date, no point mutation analogous to that in the *neu* protooncogene has been reported for human tumors. Overexpression (frequently but not uniformly due to amplification) of *erb*B2 has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King *et al.*, *Science*, 229:974 (1985); Yokota *et al.*, *Lancet*: 1:765-767 (1986); Fukushigi *et al.*, *Mol Cell Biol.*, 6:955-958 (1986); Geurin *et al.*, *Oncogene Res.*, 3:21-31 (1988); Cohen *et al.*, *Oncogene*, 4:81-88 (1989); Yonemura *et al.*, *Cancer Res.*, 51:1034 (1991); Borst *et al.*, *Gynecol. Oncol.*, 38:364 (1990); Weiner *et al.*, *Cancer Res.*, 50:421-425 (1990); Kern *et al.*, *Cancer Res.*, 50:5184 (1990); Park *et al.*, *Cancer Res.*, 49:6605 (1989); Zhau *et al.*, *Mol. Carcinog.*, 3:354-357 (1990); Aasland *et al. Br. J. Cancer* 57:358-363 (1988); Williams *et al. Pathiobiology* 59:46-52 (1991); and McCann *et al.*, *Cancer*, 65:88-92 (1990).

accelerated endocytosis of ErbB2 (see Maier *et al. Cancer Res.* 51:5361-5369 (1991)). Bacus *et al. Molecular Carcinogenesis* 3:350-362 (1990) reported that the TA1 antibody induced maturation of the breast cancer cell lines AU-565 (which overexpresses the *erb*B2 gene) and MCF-7 (which does not). Inhibition of growth and acquisition of a mature phenotype in these cells was found to be associated with reduced levels of ErbB2 receptor at the cell surface and transient increased levels in the cytoplasm.

Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991) generated a panel of anti-ErbB2 antibodies, injected them i.p. into nude mice and evaluated their effect on tumor growth of murine fibroblasts transformed by overexpression of the *erb*B2 gene. Various levels of tumor inhibition were detected for four of the antibodies, but one of the antibodies (N28) consistently stimulated tumor growth. Monoclonal antibody N28 induced significant phosphorylation of the ErbB2 receptor, whereas the other four antibodies generally displayed low or no phosphorylation-inducing activity. The effect of the anti-ErbB2 antibodies on proliferation of SKBR3 cells was also assessed. In this SKBR3 cell proliferation assay, two of the antibodies (N12 and N29) caused a reduction in cell proliferation relative to control. The ability of the various antibodies to induce cell lysis *in vitro* via complement-dependent cytotoxicity (CDC) and antibody-mediated cell-dependent cytotoxicity (ADCC) was assessed, with the authors of this paper concluding that the inhibitory function of the antibodies was not attributed significantly to CDC or ADCC.

Bacus *et al. Cancer Research* 52:2580-2589 (1992) further characterized the antibodies described in Bacus *et al.* (1990) and Stancovski *et al.* of the preceding paragraphs. Extending the i.p. studies of Stancovski *et al.*, the effect of the antibodies after i.v. injection into nude mice harboring mouse fibroblasts overexpressing human ErbB2 was assessed. As observed in their earlier work, N28 accelerated tumor growth whereas N12 and N29 significantly inhibited growth of the ErbB2-expressing cells. Partial tumor inhibition was also observed with the N24 antibody. Bacus *et al.* also tested the ability of the antibodies to promote a mature phenotype in the human breast cancer cell lines AU-565 and MDA-MB453 (which overexpress ErbB2) as well as MCF-7 (containing low levels of the receptor). Bacus *et al.* saw a correlation between tumor inhibition *in vivo* and cellular differentiation; the tumor-stimulatory antibody N28 had no effect on differentiation, and the tumor inhibitory action of the N12, N29 and N24 antibodies correlated with the extent of differentiation they induced.

Xu *et al. Int. J. Cancer* 53:401-408 (1993) evaluated a panel of anti-ErbB2 antibodies for their epitope binding specificities, as well as their ability to inhibit anchorage-independent and anchorage-dependent growth of SKBR3 cells (by individual antibodies and in combinations), modulate cell-surface ErbB2, and inhibit ligand stimulated anchorage-independent growth. See also WO94/00136 published Jan 6, 1994 and Kasprzyk *et al. Cancer Research* 52:2771-2776 (1992) concerning anti-ErbB2 antibody combinations. Other anti-ErbB2 antibodies are discussed in Hancock *et al. Cancer Res.* 51:4575-4580 (1991); Shawver *et al. Cancer Res.* 54:1367-1373 (1994); Arteaga *et al. Cancer Res.* 54:3758-3765 (1994); and Harwerth *et al. J. Biol. Chem.* 267:15160-15167 (1992).

A further gene related to *erb*B2, called *erb*B3 or HER3, has also been described. See, *e.g.*, US Pat. Nos. 5,183,884 and 5,480,968. ErbB3 is unique among the ErbB receptor family in that it possesses little or no intrinsic tyrosine kinase activity. However, when ErbB3 is co-expressed with ErbB2, an active signaling complex is formed and antibodies directed against ErbB2 are capable of disrupting this complex (Sliwkowski

the nucleic acid molecule operably linked to control sequences recognized by a host cell transformed with the vector; a host cell comprising the nucleic acid (*e.g.* a hybridoma cell line); and a process for making the antibody comprising culturing a cell comprising the nucleic acid so as to express the anti-ErbB2 antibody and, optionally, recovering the antibody from the host cell culture and, preferably, the host cell culture medium.

The invention also provides methods for using the anti-ErbB2 antibodies disclosed herein. For example, the invention provides a method for inducing cell death comprising exposing a cell, such as a cancer cell which overexpresses ErbB2, to anti-ErbB2 antibody described herein in an amount effective to induce cell death. The cell may be in cell culture or in a mammal, *e.g.* a mammal suffering from cancer. The invention also provides a method for inducing apoptosis of a cell which overexpresses ErbB2 comprising exposing the cell to exogenous anti-ErbB2 antibody as described herein in an amount effective to induce apoptosis of the cell. Thus, the invention provides a method for treating a mammal suffering from a condition characterized by overexpression of the ErbB2 receptor, comprising administering a pharmaceutically effective amount of the anti-ErbB2 antibodies disclosed herein to the mammal. According to any of the above methods, a further anti-ErbB2 antibody may be used, especially one which binds to a different ErbB2 epitope from that to which the 7C2/7F3 antibodies disclosed herein bind (*e.g.* one that does not bind Domain 1). In one embodiment, the second antibody inhibits growth of SKBR3 cells in cell culture by 50%-100% and optionally binds to the epitope on ErbB2 to which 4D5 binds.

In Example 2 below, it was found that the pro-apoptotic antibody 7C2 almost completely eradicated the entire culture of growth arrested cells. Therefore, it may be desirable to combine the pro-apoptotic antibodies disclosed herein with a growth inhibitory agent in the *in vitro* and *in vivo* methods discussed above. In such embodiments, superior levels of apoptosis may be achieved by administering the growth inhibitory agent prior to the pro-apoptotic anti-ErbB2 antibody. However, simultaneous administration or administration of the anti-ErbB2 antibody first is also contemplated.

The invention also provides an article of manufacture for use in the above *in vivo* methods which comprises a container holding the anti-ErbB2 antibody and a label on or associated with the container which indicates that the antibody can be used to treat conditions characterized by ErbB2 overexpression, such as cancer.

In a further aspect, the invention provides a method for detecting ErbB2 *in vitro* or *in vivo* comprising contacting the antibody with a cell suspected of containing ErbB2 and detecting if binding has occurred. Accordingly, the invention provides an assay for detecting a tumor characterized by amplified expression of ErbB2 comprising the steps of exposing a cell to the antibody disclosed herein and determining the extent of binding of the antibody to the cell. Preferably the antibody for use in such an assay will be labelled and will be supplied in the form of a kit with instructions for using the antibody to detect ErbB2. The assay herein may be an *in vitro* assay (such as an ELISA assay) or an *in vivo* assay. For *in vivo* tumor diagnosis, the antibody is preferably conjugated to a radioactive isotope and administered to a mammal, and the extent of binding of the antibody to tissues in the mammal is observed by external scanning for radioactivity.

**Brief Description of the Drawings**

Figs. 1A & B show the effects of apoptosis on a cell and a method for determining apoptosis, respectively. Fig. 1A shows the physiological changes which occur to a cell which undergoes programmed cell

Fig. 6 shows that the effects of MAb 7C2 are dose-dependent. The induction of apoptosis in BT474 breast tumor cells by MAb 7C2, as measured by an increase in the number of annexin V-positive and PI-positive cells, is apparent at a concentration of 0.1μg/ml and reaches a maximum at 1μg/ml.

Figs. 7A and 7B are time-courses of MAb 7C2-induced apoptosis in BT474 and SKBR3 breast tumor cells, respectively. Treatment of BT474 cells (Fig. 7A) and SKBR3 cells (Fig 7B) with 10μg/ml MAb 7C2 results in a reduction in the percent of viable cells (those cells which are annexin V- and PI-negative) as early as 15 minutes after initiation of treatment and reaches a maximum at 24 hours. The BT474 cell line is more sensitive to the pro-apoptotic effect of MAb 7C2 compared to the SKBR3 cells.

Figs 8A-E show responses of different cell lines to anti-ErbB2 MAbs. The BT474, SKBR3 and MCF7 breast tumor cell lines, and normal human mammary epithelial cells (HMEC) (Figs. 8A-D, respectively) were incubated with the anti-ErbB2 MAbs 4D5, 3H4, 7F3, 7C2, 2H11, 3E8, and 7D3; the humanized version of muMAb 4D5 (hu4D5); or the isotype-matched irrelevant control MAb 1766. Treatment was for 3 days at a MAb concentration of 10μg/ml. The data are pooled from 2-9 separate experiments and are represented as mean fold increase (+/-s.e.) in annexin V binding over control cells. The response of the BT474 breast tumor cells to MAbs 7C2 and 4D5 is as described for Fig. 5 (9-fold and 2.5-fold above control, respectively). Induction of apoptosis in the SKBR3 breast tumor cell line, which expresses high levels of ErbB2 similar to the BT474 cells, also occurs after treatment with MAb 7C2 (and to a smaller degree, MAb 4D5). In addition, MAb 7F3 induces apoptosis in both the BT474 and SKBR3 cell lines. The MCF7 breast tumor line, which expresses normal ErbB2 levels, and the HMEC's showed no change in annexin V binding after treatment with the anti-ErbB2 Mabs. These results suggest that overexpression of ErbB2 is required for responsiveness to the anti-ErbB2 MAbs. In Fig. 8E, a non-small cell lung carcinoma line overexpressing ErbB2, Calu 3, was tested for induction of apoptosis by anti-ErbB2 MAbs. Treatment with 7C2 or 7F3 resulted in enhanced binding of annexin V.

Figs. 9A-I show the effects of MAbs 7C2 and 4D5 on cell cycle progression and cell death. Untreated BT474 cells are largely annexin V-negative and PI-negative (Fig. 9A, quadrant 3), and show a normal cell cycle DNA histogram (Figs. 9B &C). Cells treated with 10μg/ml MAb 4D5 show some increase in uptake of PI and annexin V-FITC binding (Fig. 9D, quadrant 2). The most pronounced effect is on cell cycle progression, where MAb 4D5 almost completely reduces the percent of cells in S phase (Figs. 9E &F). MAb 7C2 induces a significant amount of cell death in BT474, as measured by PI uptake and annexin V-FITC binding (Fig. 9G, quadrant 2). Cell cycle analysis shows the presence of a sub-$G_0/G_1$ or hypodiploid population (Fig. 9I), characteristic of apoptotic cells, with the cells displaying high levels of annexin V binding (Fig. 9H, quadrant 1).

Figs. 10A-F are the results from curve-fitting analyses of the DNA histograms of Figs. 9A-I and show little change in the percent of cells in S-phase (52%) after MAb 7C2 treatment compared to control cells (61% S-phase cells), but a dramatic reduction in the number of cells in S-phase (to 6%) in response to MAb 4D5 (Figs. 10C, A and B, respectively). Analyses of the apoptotic population of cells (annexin V/PI positive cells from quadrant 2, 9A, D and G) reveal no difference in the percent S-phase cells compared to the total cell population (Fig. 10D control=55%; Fig. 10E MAb 4D5=7%; Fig. 10F MAb 7C2=56%). Furthermore, the

53.4%, respectively, of the cell population), but did not decrease the proportion of S-phase cells (64.9% and 58.7%, respectively) compared to untreated cells (52.5%; Fig. 14A). In contrast, MAbs 4D5 and 3H4, which bind adjacent to the ErbB2 transmembrane region, show potent anti-proliferative activity (%S=5.4 and 10.5, respectively, control S=52.5%), but are not as effective as 7C2 or 7F3 in promoting apoptotic cell death (% apoptosis for 4D5=41.9, for 3H4=26.3, controls=15.8%).

Fig. 15 shows induction of apoptosis by anti-HER2 MAb 7C2 in the SKOV3 ovarian carcinoma cell line as determined in Example 3.

Fig. 16 shows that combination treatment with anti-HER2 MAbs results in enhanced apoptotic effects on BT474 breast tumor cells where anti-HER2 MAb 7C2 is administered prior to anti-HER2 MAb 4D5 (see Example 3).

Fig. 17 shows the effects of administration of anti-HER2 MAbs alone or in combination on mean tumor volume ($mm^3$) +/- 1 S.E. of BT474M1 xenografts in nude mice as described in Example 4. Antibodies were administered twice weekly beginning on day 6.

## Detailed Description of the Preferred Embodiments

### I. Definitions

Unless indicated otherwise, the term "ErbB2" when used herein refers to human ErbB2 protein and "*erb*B2" refers to human *erb*B2 gene. The human *erb*B2 gene and ErbB2 protein are described in Semba *et al.*, *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto *et al. Nature* 319:230-234 (1986) (Genebank accession number X03363), for example. ErbB2 comprises four domains (Domains 1-4). "Domain 1" at the amino terminus of the extracellular domain of ErbB2 is shown in Fig. 12 herein. See Plowman *et al. Proc. Natl. Acad. Sci USA* 90:1746-1750 (1993).

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see Example 2 below) to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2 (*i.e.* any one or more of residues in the region from about residue 22 to about residue 53 of ErbB2 (SEQ ID NO:2)).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed (see Example 2 below) to assess whether the antibody binds to the 4D5 epitope of ErbB2 (*i.e.* any one or more residues in the region from about residue 529, *e.g.* about residue 561 to about residue 625, inclusive (SEQ ID NO:4)).

The term "induces cell death" refers to the ability of the antibody to make a viable cell become nonviable. The "cell" here is one which expresses the ErbB2 receptor, especially where the cell overexpresses the ErbB2 receptor. A cell which "overexpresses" ErbB2 has significantly higher than normal ErbB2 levels compared to a noncancerous cell of the same tissue type. Preferably, the cell is a cancer cell, *e.g.* a breast,

*Science*, 256:1205-1210 (1992); WO 92/20798; Wen *et al.*, *Mol. Cell. Biol.*, 14(3):1909-1919 (1994); and Marchionni *et al.*, *Nature*, 362:312-318 (1993), for example. The term includes biologically active fragments and/or variants of a naturally occurring HRG polypeptide, such as an EGF-like domain fragment thereof (*e.g.* $HRG\beta1_{177-244}$).

The "ErbB2-ErbB3 protein complex" and "ErbB2-ErbB4 protein complex" are noncovalently associated oligomers of the ErbB2 receptor and the ErbB3 receptor or ErbB4 receptor, respectively. The complexes form when a cell expressing both of these receptors is exposed to HRG and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski *et al.*, *J. Biol. Chem.*, 269(20):14661-14665 (1994).

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain ($V_H$) followed by a number of constant domains. Each light chain has a variable domain at one end ($V_L$) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light- chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *NIH Publ. No.91-3242*, Vol. I, pages 647-669 (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen- binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al., Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature*, 321:522-525 (1986); Reichmann *et al., Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a PRIMATIZED™antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the $V_H$ and $V_L$ domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the $V_H$ and $V_L$ domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain ($V_H$) connected to a light-chain variable domain ($V_L$) in the same polypeptide chain ($V_H$ - $V_L$). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain

A "chemotherapeutic agent" is a chemical c mpound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytoxin, Taxol, Toxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincreistine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially an ErbB2-overexpressing cancer cell either *in vitro* or *in vivo*. Thus, the growth inhibitory agent is one which significantly reduces the percentage of ErbB2 overexpressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxol, and topo II inhibitors such as doxorubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic drugs" by Murakami *et al.* (WB Saunders: Philadelphia, 1995), especially p. 13. The 4D5 antibody (and functional equivalents thereof) can also be employed for this purpose.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-$\alpha$ and -$\beta$; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-$\beta$; platelet-growth factor; transforming growth factors (TGFs) such as TGF-$\alpha$ and TGF-$\beta$; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-$\alpha$, -$\beta$, and -$\gamma$; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1$\alpha$, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-$\alpha$ or TNF-$\beta$; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting

"operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

## II. Modes for Carrying out the Invention

### A. Antibody Preparation

A description follows as to exemplary techniques for the production of the claimed antibodies. The ErbB2 antigen to be used for production of antibodies may be, *e.g.*, a soluble form of the extracellular domain of ErbB2; a peptide such as a Domain 1 peptide or a portion thereof (*e.g.* comprising the 7C2 or 7F3 epitope). Alternatively, cells expressing ErbB2 at their cell surface (*e.g.* NIH-3T3 cells transformed to overexpress ErbB2, see Examples 1 & 2 below; or a carcinoma cell line such as SKBR3 cells, see Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of ErbB2 useful for generating antibodies will be apparent to those skilled in the art.

#### *(i) Polyclonal antibodies*

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and $R^1$ are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

#### *(ii) Monoclonal antibodies*

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al., Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al., Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

*(iii) Humanized and human antibodies*

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al., Nature*, 321:522-525 (1986); Riechmann *et al., Nature*, 332:323-327 (1988); Verhoeyen *et al., Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

*(v) Bispecific antibodies*

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the ErbB2 protein. For example, one arm may bind an epitope in Domain 1 of ErbB2 such as the 7C2/7F3 epitope, the other may bind a different ErbB2 epitope, *e.g.* the 4D5 epitope. Other such antibodies may combine an ErbB2 binding site with binding site(s) for EGFR, ErbB3 and/or ErbB4. Alternatively, an anti-ErbB2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express ErbB2. These antibodies possess an ErbB2-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al., EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted

heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain ($V_H$) connected to a light-chain variable domain ($V_L$) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the $V_H$ and $V_L$ domains of one fragment are forced to pair with the complementary $V_L$ and $V_H$ domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

*(vi) Screening for antibodies with the desired properties*

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, *e.g.*, PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection (Rockville, MD)) are cultured in Dulbecco's Modified Eagle Medium (D-MEM):Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3 x $10^6$ per dish in 100 x 20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200rpm for 5 minutes at $4^0$C, the pellet resuspended in 3 ml ice cold $Ca^{2+}$ binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1ml per tube, 3 tubes per treatment group) for removal of cell clumps. Tubes then receive PI (10μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" as described in Example 2 below is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10μg/ml of the MAb. Following a three day incubation period; monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in $Ca^{2+}$ binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labelled annexin (*e.g.* annexin V-FTIC) (1μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to contr l are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay discussed in the preceding paragraph, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al. Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.* avidin) which is conjugated to a cytotoxic agent (*e.g.* a radionucleotide).

*(ix) Immunoliposomes*

The anti-ErbB2 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.* 81(19)1484 (1989)

*(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)*

The antibody of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as ß-galactosidase and neuraminidase

consisting of HQSLGTQ (SEQ ID NO:6), HQNLSDGK (SEQ ID NO:7), HQNISDGK (SEQ ID NO:8), or VISSHLGQ (SEQ ID NO:9), particularly where the antibody fragment is a Fab or $F(ab')_2$. In an ther most preferred embodiment, the salvage receptor binding epitope is a polypeptide containing the sequence(s)(5' to 3'): HQNLSDGK (SEQ ID NO:7), HQNISDGK (SEQ ID NO:8), or VISSHLGQ (SEQ ID NO:9) and the sequence: PKNSSMISNTP (SEQ ID NO:5).

**B. Vectors, Host Cells and Recombinant Methods**

The invention also provides isolated nucleic acid encoding an antibody as disclosed herein, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody. In addition to recombinant production of the antibody, the nucleic acid encoding the antibodies disclosed herein may be used to inhibit cell surface expression of the ErbB2 protein according to the teachings of WO96/07321, published March 14, 1996, for example. For example, the antibody may be a single chain Fv fragment provided in an expression vector (such as a viral or plasmid vector), which vector is introduced into a cell so as to bind to the ErbB2 protein intracellularly and thereby induce death of the cell.

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components preferably include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) *Signal sequence component*

The anti-ErbB2 antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native anti-ErbB2 antibody signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the anti-ErbB2 antibody.

*(ii) Origin of replication component*

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Preferably, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or

*(iv) Promoter component*

Expression and cloning vect rs usually contain a promoter that is recognized by the host organism and is operably linked to the anti-ErbB2 antibody nucleic acid. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter , β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the anti-ErbB2 antibody.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Anti-ErbB2 antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature*, 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.*, 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for anti-ErbB2 antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

*(viii) Culturing the host cells*

The host cells used to produce the anti-ErbB2 antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al. Meth. Enz.*, 58:44 (1979), Barnes *et al.*, *Anal. Biochem.*,102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to EGFR, ErbB2 (*e.g.* an antibody which binds a different epitope on ErbB2), ErbB3, ErbB4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™(injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

**D. Non-therapeutic Uses for the Antibody**

The antibodies of the invention may be used as affinity purification agents. In this process, the antibodies are immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known

(iii) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (*e.g.* p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with a small hapten (*e.g.* digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.* anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-ErbB2 antibody need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the ErbB2 antibody.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of ErbB2 protein in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies preferably are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

The antibodies may also be used for *in vivo* diagnostic assays. Preferably, the antibody is labelled with a radionuclide (such as $^{111}In$, $^{99}Tc$, $^{14}C$, $^{131}I$, $^{125}I$, $^{3}H$, $^{32}P$ or $^{35}S$) so that the tumor can be localized using immunoscintiography.

**E. Diagnostic Kits**

As a matter of convenience, the antibody of the present invention can be provided in a kit, *i.e.*, a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labelled with an enzyme, the kit will include substrates and cofactors required

For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (*e.g.* 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

**G. Articles of Manufacture**

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the anti-ErbB2 antibody. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

**H. Deposit of Materials**

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

| Antibody Designation | ATCC No. | Deposit Date |
|---|---|---|
| 7C2 | ATCC HB-12215 | October 17, 1996 |
| 7F3 | ATCC HB-12216 | October 17, 1996 |
| 4D5 | ATCC CRL 10463 | May 24, 1990 |

These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of deposit. The cell lines will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures (a) that access to the cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR §1.14 and 35 USC §122, and (b) that all restrictions on the availability to the public of the cultures so deposited will be irrevocably removed upon the granting of the patent.

dye which stains all permeable cells. Cells were then fixed with 1.0ml of 0.5% paraformaldehyde in PBS and simultaneously permeabilized and stained for 16 hours at 4°C with 220µl of 10µg/ml HOECHST 33342™ dye (also a DNA binding dye) containing 5% TWEEN 20™.

The data from 1 x $10^4$ cells were collected and stored using LYSYS II™ software and analyzed using PAINT-A-GATE™ software (Becton Dickinson) (Darzynkiewica *et al. Cytometry* 13:795-808 (1992) and Picker *et al. J. Immunol.* 150(3):1105-1121 (1993)). The viability and percentage of cells in each stage of the cell cycle were determined on gated single cells using 7AAD and Hoechst staining, respectively. (Cell doublets were excluded by pulse analysis of width vs. area of the Hoechst signal.) Cell numbers were determined using a hemocytometer.

**DNA synthesis.** Triplicate cultures of 8 x $10^3$ cells/well were plated in 96-well flat bottom plates, allowed to adhere overnight, then continuously incubated in the presence or absence of anti-ErbB2 or control Ig for different periods of time. During the last 12 hours of culture, wells were pulsed with 1µCi $^3$H-thymidine (Amersham, Arlington, VA).

**Affinity of binding to the extracellular domain of the ErbB2.** Hadioiodinated anti-ErbB2 antibodies were prepared by the Iodogen method (Fracker *et al. Biochem. Biophys. Res. Comm.* 80:849-857 (1978)). Binding assays were performed using monolayers of BT474 cells cultured in 96-well tissue culture plates (Falcon, Becton Dickenson Labware, Lincoln Park, N.J.). The cells were trypsinized and seeded in wells of 96-well plates at a density of $10^4$ cells/well and allowed to adhere overnight. The monolayers were washed with cold culture medium supplemented with 0.1% sodium azide and then incubated in triplicate with 100µl of serial dilutions of $^{125}$I-anti-ErbB2 antibodies in cold culture medium with 0.1% azide for 4 hours on ice. Non-specific binding was estimated by the preincubation of each sample with a 100-fold molar excess of nonradioactive antibodies in a total volume of 100µl. Unbound radioactivity was removed by two washes with cold medium with 0.1% sodium azide. The cell-associated radioactivity was detected in a gamma counter after solubilization of the cells with 150µl 0.1 M NaOH/well. The anti-ErbB2 binding constants ($K_d$) were determined by Scatchard analysis.

**Results.** The binding affinities of anti-ErbB2 antibodies (7C2 and 4D5) were determined by Scatchard analysis. The binding constants ($K_d$) were 6.5 x $10^{-9}$ M (4D5) and 2.9 x $10^{-9}$ M (7C2). Blocking experiments were carried out using unlabelled antibodies followed by FITC-7C2. As shown in Fig. 2, 4D5 reacts with a different epitope than 7C2.

The effect of these antibodies on the growth of the BT474 human breast cancer cells which overexpress ErbB2 was then investigated. Fig. 3A shows the results of flow cytometric analysis of cells incubated with an isotype-matched control. 10-12% of the cells were dead and 28% of the viable cells were in the S-$G_2$-M phases of the cell cycle. Similar results were obtained when the cells were incubated in medium alone. Treatment with 4D5 (Fig. 3B) induced a decrease in cell size as measured by forward light scatter, a moderate increase in the proportion of dead cells (27.0%) and a marked decrease of viable cells in S-$G_2$-M (6.3%) with a concurrent increase of cells in $G_0/G_1$ (94%) as compared to the control cells. Cell counts were reduced by 46.7%. Without being bound to any one theory, it appears that 4D5 induces primarily cell cycle arrest (CCA) in $G_0/G_1$ but that a significant proportion of cells also die. Fig. 3C shows the results of incubating the BT474 cell with 50µg/ml of 7C2. There was no change in forward light scatter of the residual

0.5, 1, 2, 24, 72, 96 hr, 7 or 10 days. MAb concentrations used in the dose-response experiments were 0.01, 0.1, 1 and 10μg/ml. Following each treatment, supernatants were individually collected and kept on ice, monolayers were detached by trypsinization and pooled with the corresponding supernatant. Cells were then centrifuged at 1200rpm for 5 minutes at $4^0$C, the pellet resuspended in 3 ml ice cold $Ca^{2+}$ binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1ml per tube, 3 tubes per treatment group) for removal of cell aggregates. Each group of 3 tubes then received annexin V-FTIC (1μg/ml) or PI (10μg/ml) or annexin V-FTIC plus PI. Samples were analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). For cell cycle analysis, cells were incubated with 9μg/ml HOECHST 33342™ for 2 hr at $37^0$C, then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House).

Serum-deprivation experiments were performed in the following way. BT474 breast tumor cells were seeded in culture medium at a density of 5 x $10^6$ per dish in 100 x 20 mm dishes. The following day, the medium was replaced with medium containing 0.1% FBS and the cells were incubated for 3 days. Cells then received 10μg/ml of MAb 7C2 or 4D5 in fresh medium supplemented with 0.1% FBS. After a 3 day incubation, analyses of annexin V binding, PI uptake and cell cycle progression were performed as described above. In order to compare growth of serum-starved cells to non-deprived cells, separate dishes of BT474 cells, incubated in medium supplemented with 10% FBS for all time points, were studied in parallel.

**Detection of DNA ladder formation.** For measuring internucleosomal fragmentation of DNA, BT474 breast tumor cells were plated and treated for 3 days with 10 μg/ml MAb 4D5 or 7C2 as described above. DNA was extracted, $^{32}$P-end-labeled, and run on a 2% agarose gel containing 5μg/ml ethidium bromide. The gel was then dried and exposed to Kodak film. The formation of DNA ladders, a hallmark of apoptosis, was observed in BT474 breast tumor cells treated with 10μg/ml MAb 7C2 or MAb 4D5 for 3 days.

**Electron micrography studies.** BT474 cells were treated with 10μg/ml MAb 7C2 for 3 days, then fixed in 1.25% formaldehyde/1% glutaraldehyde in 0.1M cacodylate buffer. Post-fixation was performed in 2% osmium tetroxide in cacodylate buffer. The fixed cells were then end-block stained in uranyl acetate, dehydrated in graded concentrations of ethanol, and embedded in Eponet. Sections were cut utilizing a microtome and observed under a Philips CM12™ electron microscope. Highly shrunken cells displaying nuclear and cytoplasmic condensation, typical of apoptotic cells, were observed after treatment with MAb 7C2. The apoptotic cells eventually become phagocytosed by underlying cells.

The results of the experiments performed are shown in Figs. 5-14. Certain anti-ErbB2 MAbs induce apoptosis in human tumor cell lines which overexpress ErbB2 as evidenced by electron microscopy, annexin-V binding, cell cycle analysis of DNA content, DNA laddering and time-lapse videomicrography. Anti-ErbB2 MAbs 7C2 and 7F3, which recognize the same epitope on the ErbB2 extracellular domain, display the most potent pro-apoptotic effects. Anti-ErbB2 MAb 4D5, which recognizes a different ErbB2 epitope, induces a small amount of apoptosis in addition to its potent reduction in proliferation. Induction of apoptotic cell death by 7C2 or 4D5 appears to be independent of cell cycle. Inhibition of growth, either by serum deprivation or by treatment with 4D5, followed by treatment with 7C2 can result in complete cell death of the culture.

were inoculated by subcutaneous injection with 5 million BT474M1 tumor cells in MATRIGEL™ 24 hours after estrogen implantation. Animals were monitored daily for well being and tumors were measured twice weekly. Reports on tumor measurements were supplied as they were collected. Animals were weighed weekly to assess toxicity during the study. One hundred five (105) animals were inoculated and all animals were evaluated in the study.

**Treatment Protocol**. Animals were randomized to one of 7 treatment groups (15 animals per group). Treatment was initiated 6 days after inoculation of tumors. Tumor sizes for all animals and mean tumor sizes for each of the treatment groups were examined prior to beginning treatment to ensure consistency between groups. Treatment groups were:

1) Vehicle control injection - 100 µl by intraperitoneal (IP) injection twice weekly
2) Irrelevant antibody (αgp120) (isotype matched) - 10mg/kg in 100 µl IP twice weekly
3) MAb 7C2 - 10 mg/kg in 100 µl by IP injection twice weekly
4) MAb 7F3 - 10 mg/kg in 100 µl by IP injection twice weekly
5) MAb 4D5 - 10 mg/kg in 100 µl by IP injection twice weekly
6) MAb 7C2 (10mg/kg) + MAb 4D5 (10 mg/kg) - in 100 µl by IP injection weekly
7) MAb 7F3 (10 mg/kg) + MAb 4D5 (10 mg/kg) - in 100 µl by IP injection weekly

All treatment groups were treated twice weekly for a total of 10 treatments by intraperitoneal injection. Treatment groups 1-4 were euthanized at this point due to large tumor size. Treatment groups 5, 6 and 7 were continued and received a total of 16 antibody treatments. Throughout the study, animals were monitored daily for well being, twice weekly for tumor measurements and weekly for body weights. Individual animals data and mean data by treatment group was supplied as it became available.

**Termination of experiment**. After conclusion of treatment, animals in treatment groups 1-4 were euthanized due to some large tumor sizes within these treatment groups. Tumor volume was not allowed to exceed 4gms (4,000 $mm^3$). No animals were observed with significant weight loss and weight loss greater than 15% loss of body weight from the weight at onset of treatment was never observed. At the conclusion of the experiment, all animals were euthanized.

**Results**. The monoclonal antibodies, 4D5, 7C2 and 7F3, directed against the HER2 growth factor receptor, were used in a mouse xenograft model which overexpresses the HER2 receptor. Antibodies were used alone and in combinations of growth inhibiting antibody (4D5) with apoptotic antibodies (7C2 and 7F3). The apoptotic antibodies (7C2 and 7F3) had a growth inhibitory effect early on in the study that was lost at later time points (Fig. 17). The growth inhibitory antibody, 4D5, had a marked growth inhibitory effect throughout the study, as has been reported in previous studies. The combination of 4D5 with either 7C2 or 7F3 potentiated the growth inhibitory effect significantly, with 4D5/7C2 being the best combination. There was one complete remission in the 4D5 alone treatment group and one complete remission in the 4D5/7C2 treatment group. The antibody control group (anti-gp120 MAb) was equivalent to the saline treated control group. Body weights initially increased after tumor inoculation, then were maintained through the remainder of the study. None of the antibodies exhibited any toxic effect on the animals.

Ile Ala His Asn Gln Val Arg Gln Val Pro Leu Gln Arg Leu Arg
65 70 75

Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr Ala Leu Ala
80 85 90

Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro Val Thr
95 100 105

Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser Leu
110 115 120

Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
125 130 135

Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys
140 145 150

Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg
155 160 165

Ala
166

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Thr Gln Val Cys Thr Gly Thr Asp Met Lys Leu Arg Leu Pro
1 5 10 15

Ala Ser Pro Glu Thr His Leu Asp Met Leu Arg His Leu Tyr Gln
20 25 30

Gly Cys
32

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
1 5 10 15

Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
20 25 30

Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

His Gln Asn Leu Ser Asp Gly Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

His Gln Asn Ile Ser Asp Gly Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val Ile Ser Ser His Leu Gly Gln
1 5 8

28. A method for inducing cell death comprising exposing a cell which overexpresses ErbB2 to an effective amount of the antibody of claim 1.

29. The method of claim 28 wherein the cell is a cancer cell.

30. The method of claim 28 wherein the cell is in a mammal.

31. The method of claim 30 wherein the mammal is a human.

32. The method of claim 28 further comprising exposing the cell to a second anti-ErbB2 antibody which does not bind to Domain 1 of ErbB2.

33. The method of claim 28 further comprising exposing the cell to a second antibody which binds ErbB2 and inhibits growth of SKBR3 cells in cell culture by 50-100%.

34. The method of claim 33 wherein the cell is exposed to the antibody which binds to Domain 1 of ErbB2 before the cell is exposed to the second antibody.

35. The method of claim 33 wherein the second antibody binds to epitope 4D5 on ErbB2.

36. The method of claim 35 wherein the second antibody has complementarity determining regions (CDRs) of antibody 4D5.

37. The method of claim 28 further comprising exposing the cell to a growth inhibitory agent.

38. The method of claim 28 further comprising exposing the cell to a chemotherapeutic agent.

39. The method of claim 28 further comprising exposing the cell to radiation.

40. A method for inducing cell death comprising exposing a cell which overexpresses ErbB2 to an effective amount of the antibody of claim 9.

41. An article of manufacture, comprising:

a container;

a label on the container; and

a composition comprising an active agent contained within the container; wherein the composition is effective for inducing cell death, the label on the container indicates that the composition can be used for treating conditions characterized by overexpression of ErbB2 and the active agent in the composition is the antibody of claim 1.

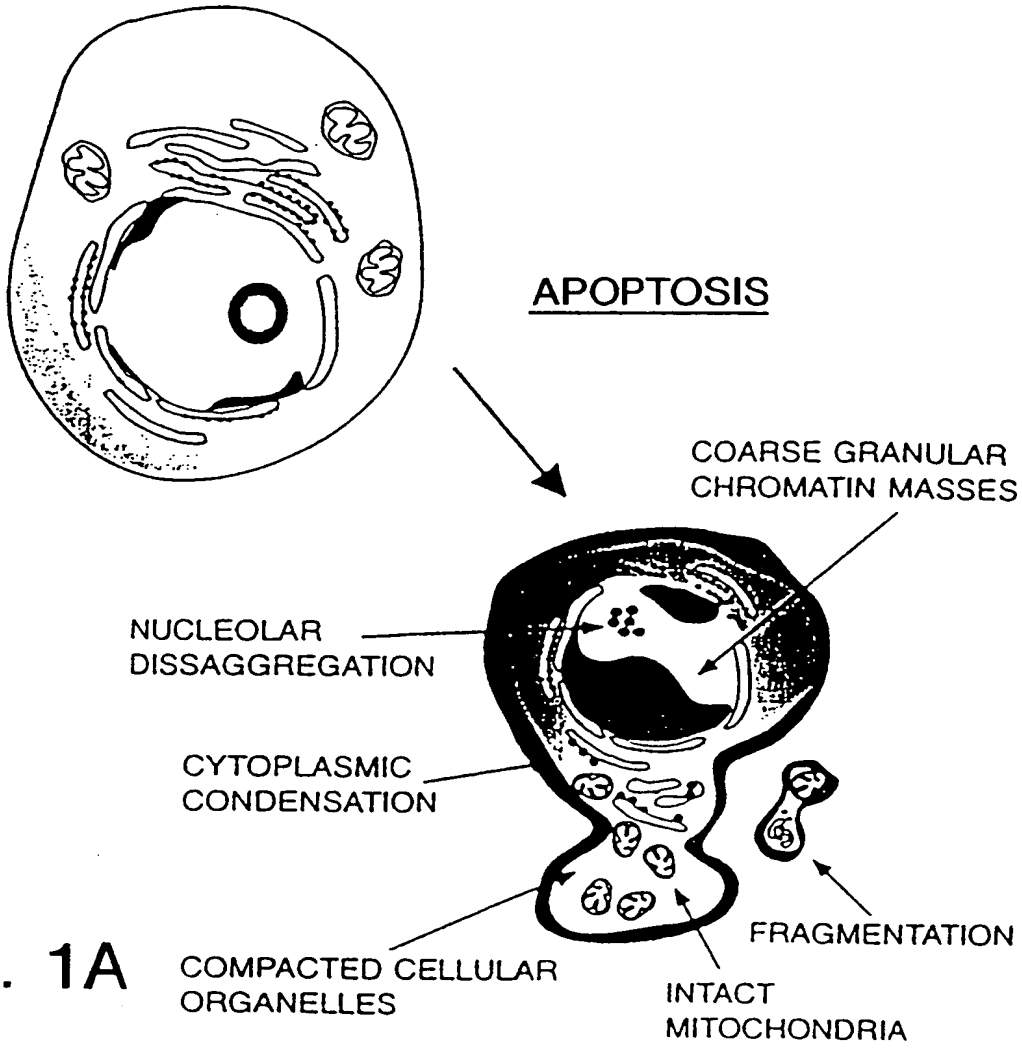

APOPTOSIS
COARSE GRANULAR
CHROMATIN MASSES
NUCLEOLAR
DISSAGGREGATION
CYTOPLASMIC
CONDENSATION
FRAGMENTATION
COMPACTED CELLULAR
ORGANELLES
INTACT
MITOCHONDRIA

FIG. 1A

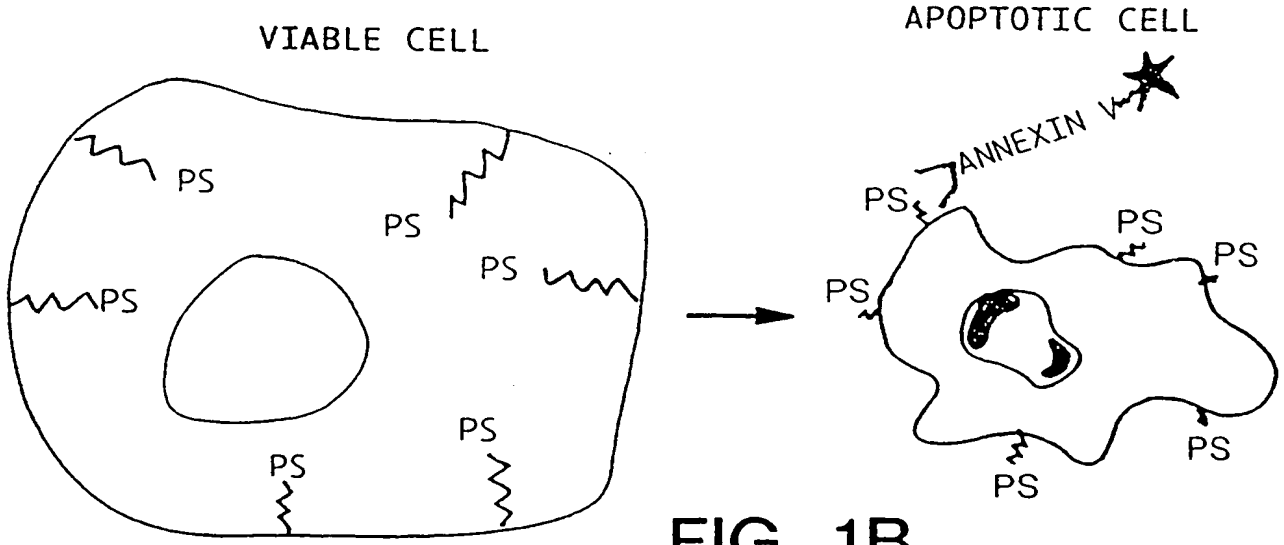

VIABLE CELL
APOPTOTIC CELL
PS
PS
PS
PS
PS
PS
PS
PS
ANNEXIN V
PS
PS
PS
PS
PS
PS

FIG. 1B

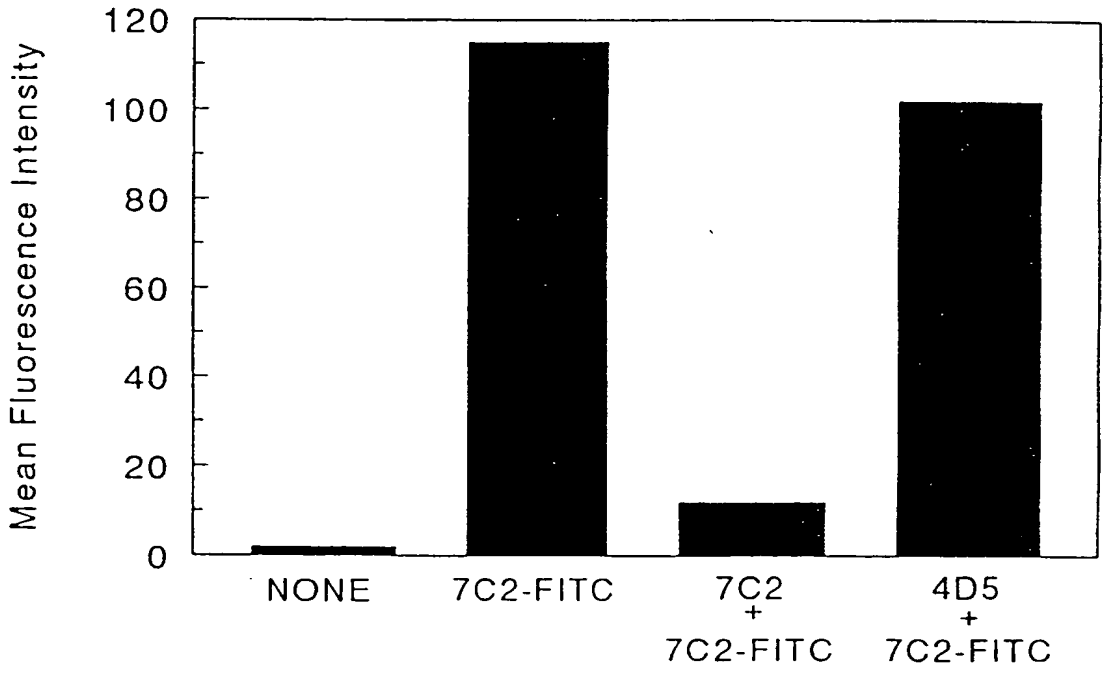

Mean Fluorescence Intensity
120
100
80
60
40
20
0
NONE
7C2-FITC
7C2 + 7C2-FITC
4D5 + 7C2-FITC

FIG. 2

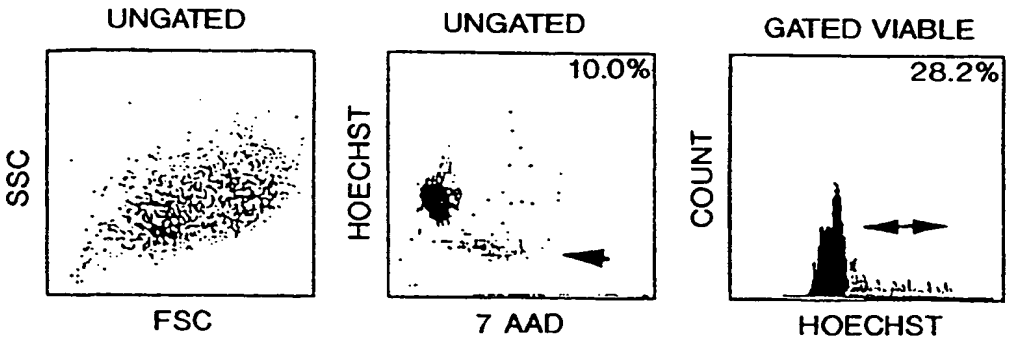



FIG. 3A

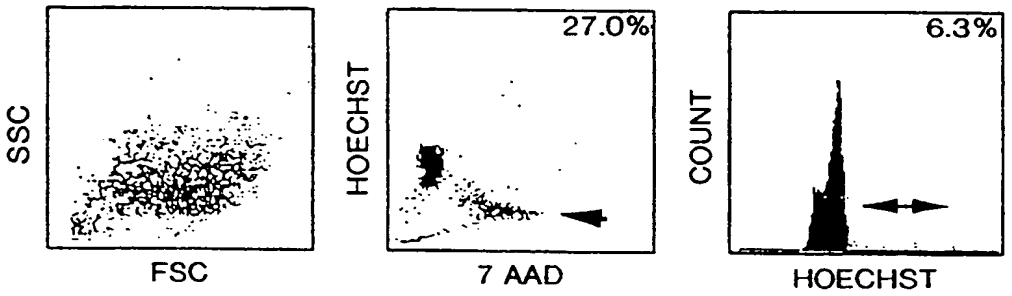



FIG. 3B

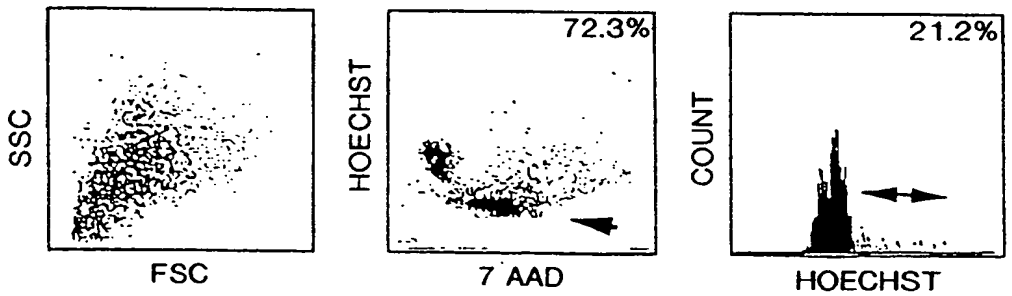



FIG. 3C

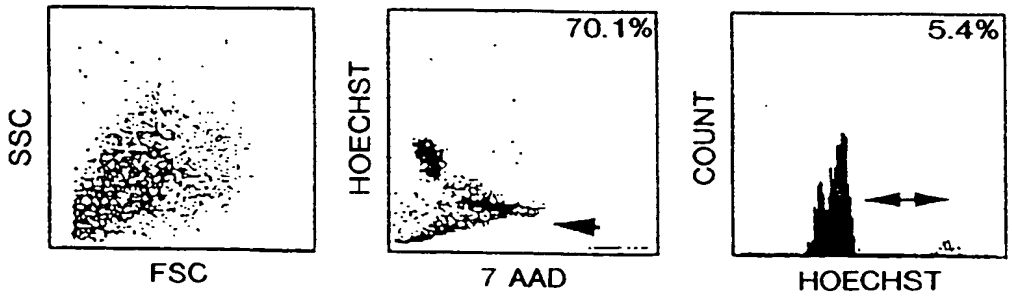



FIG. 3D

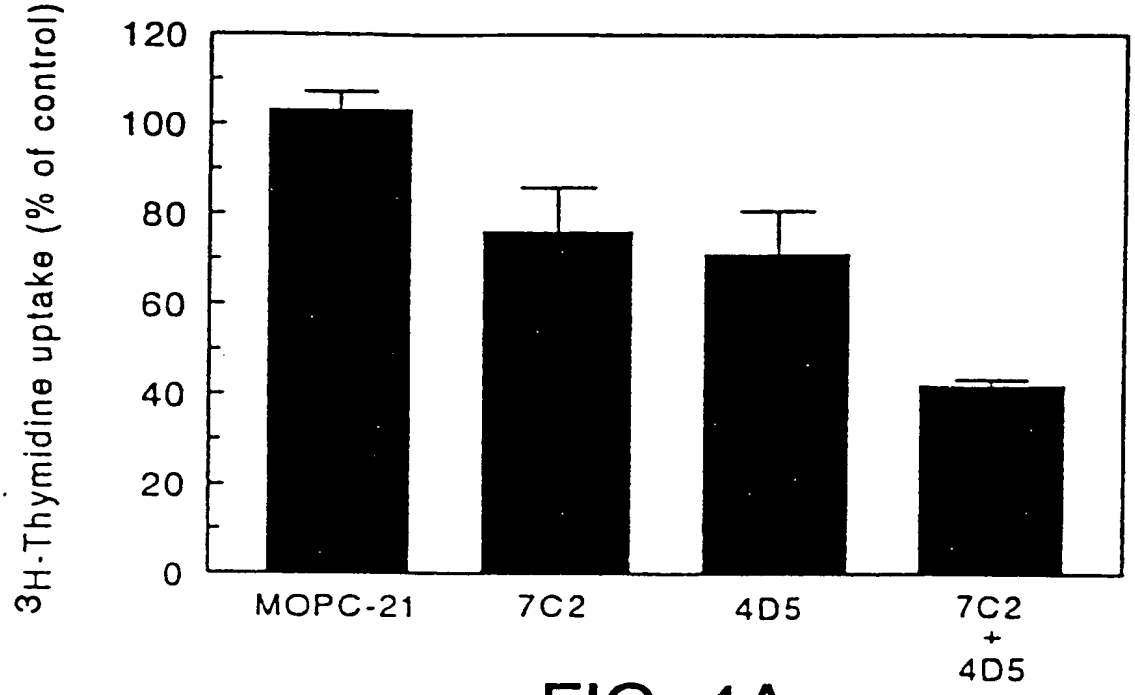

$^3$H-Thymidine uptake (% of control)
120
100
80
60
40
20
0
MOPC-21
7C2
4D5
7C2 + 4D5

FIG. 4A

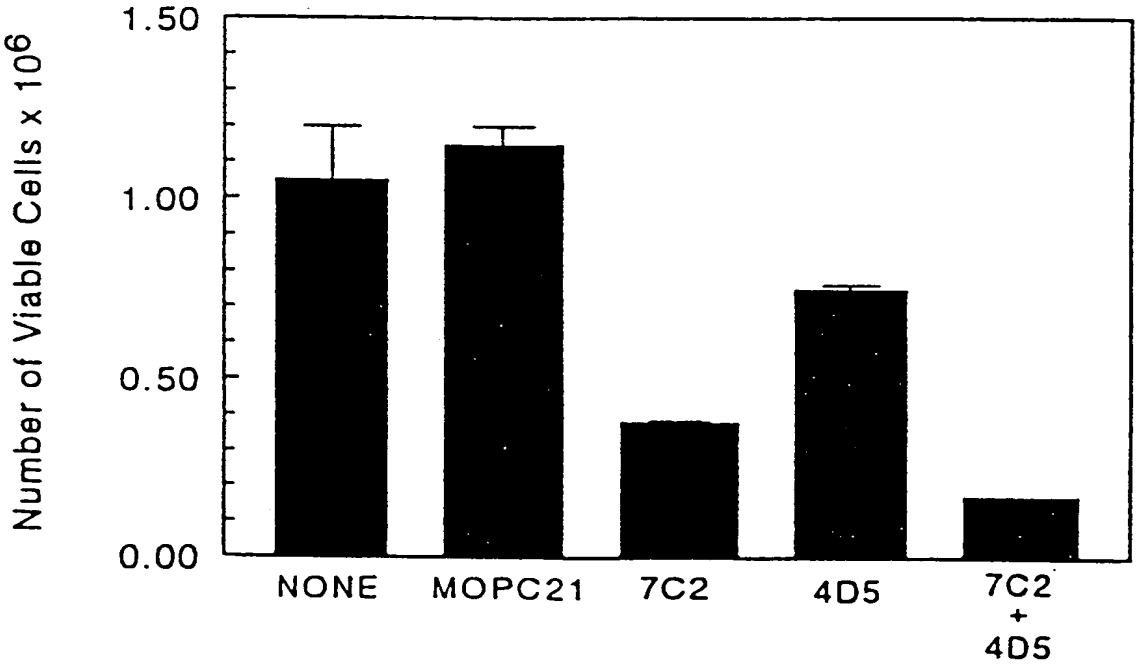

Number of Viable Cells x $10^6$
1.50
1.00
0.50
0.00
NONE
MOPC21
7C2
4D5
7C2 + 4D5

FIG. 4B

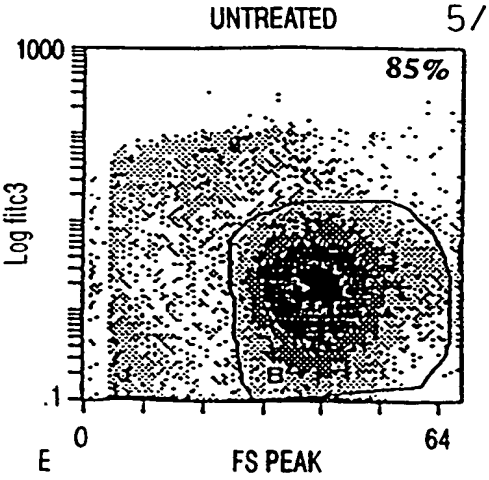



FIG. 5A

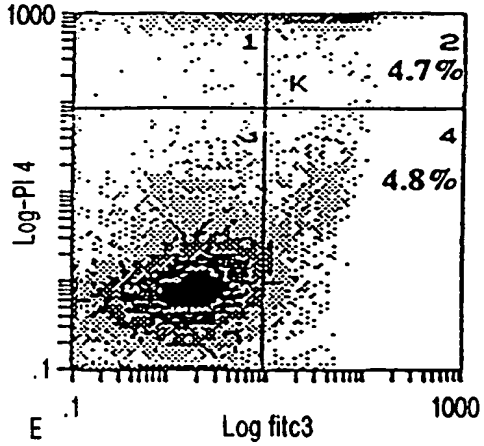



FIG. 5B

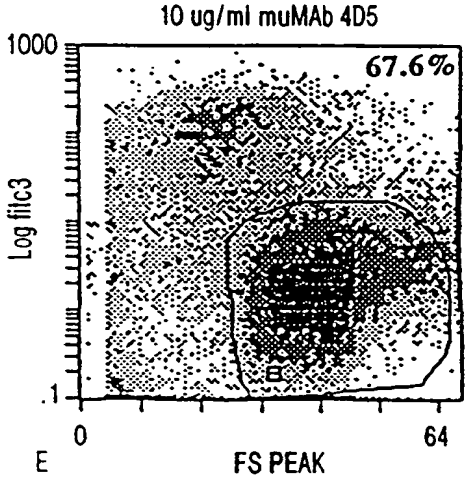



FIG. 5C

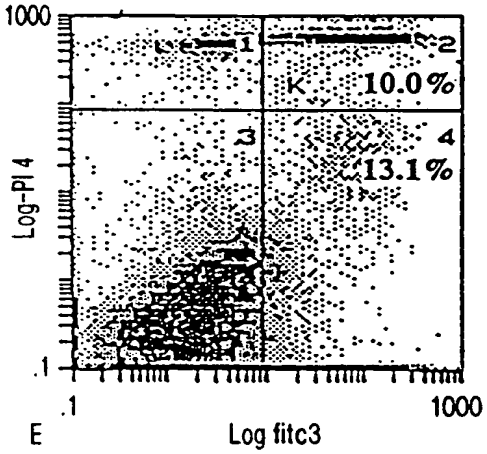



FIG. 5D

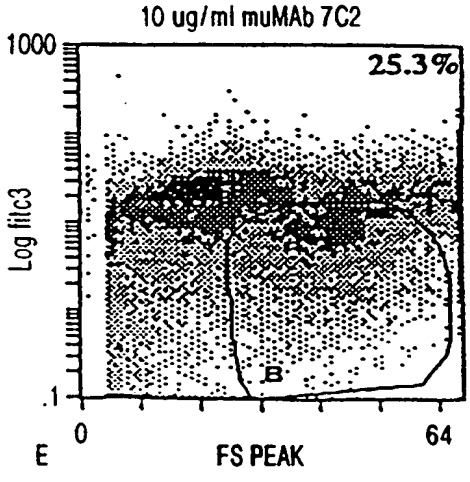



FIG. 5E

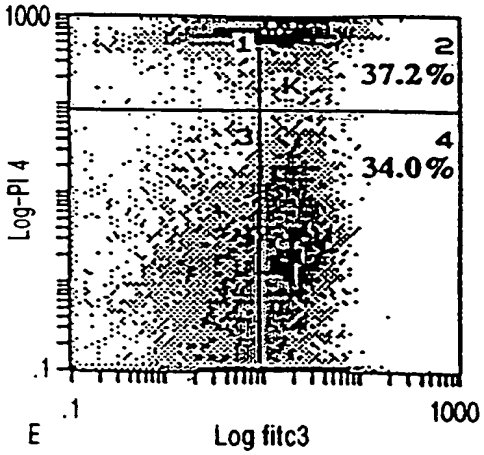



FIG. 5F

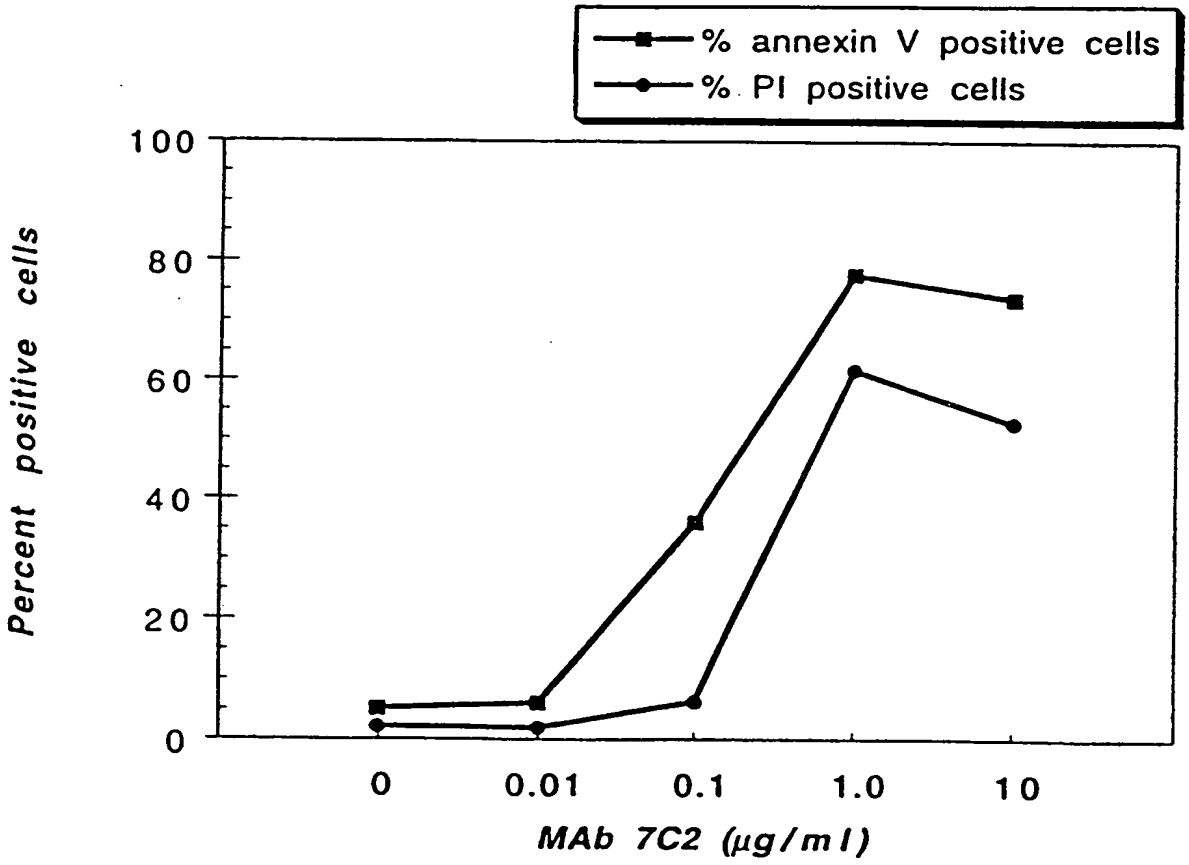

% annexin V positive cells
% PI positive cells
Percent positive cells
100
80
60
40
20
0
0
0.01
0.1
1.0
10
MAb 7C2 (µg/ml)

FIG. 6

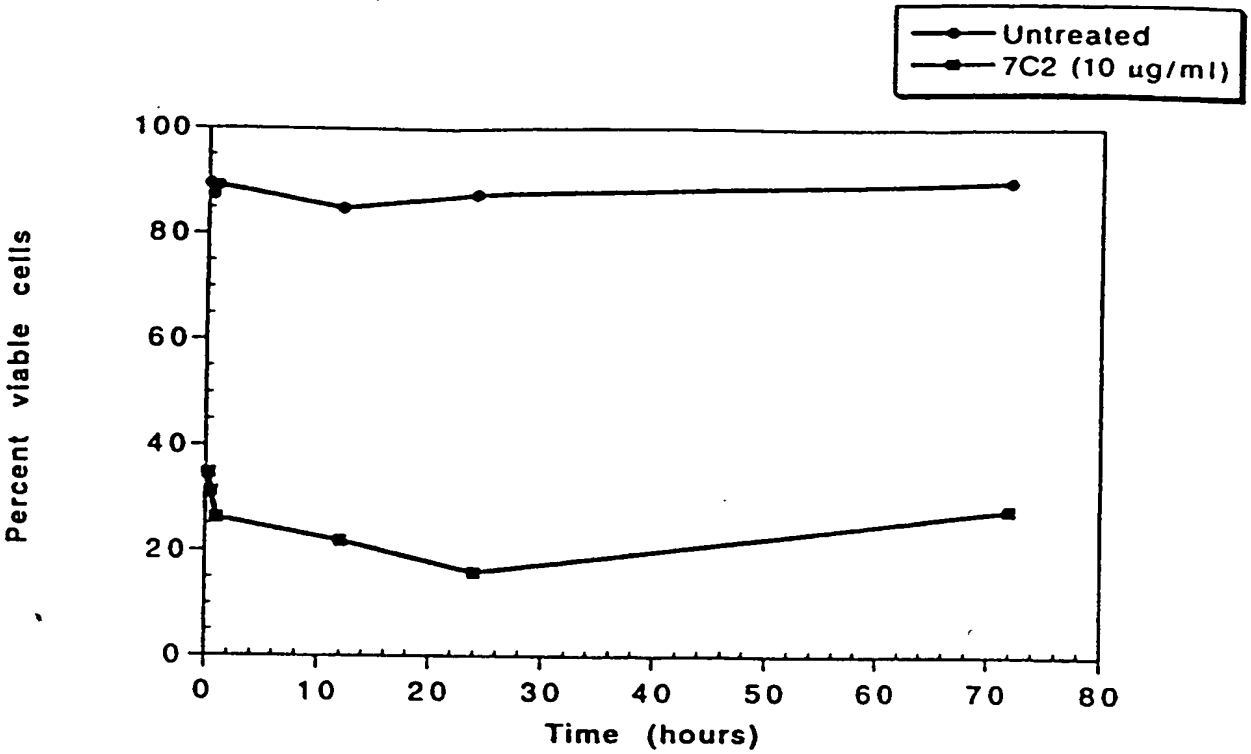

Untreated
7C2 (10 ug/ml)
Percent viable cells
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Time (hours)

FIG. 7A

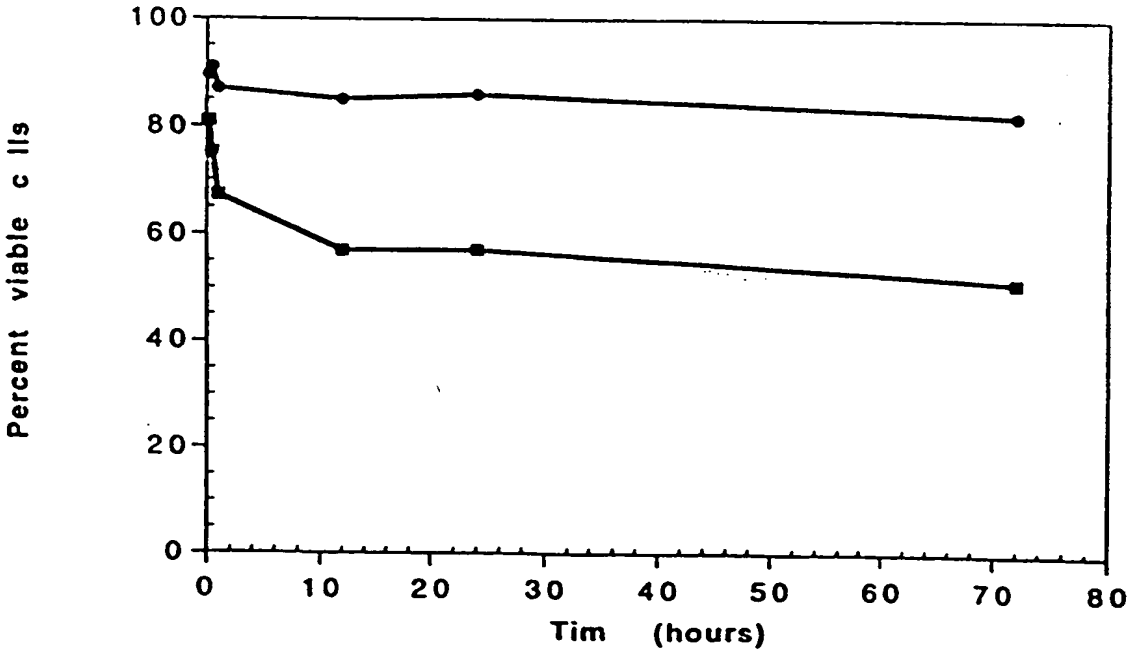

Percent viable c lls
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Tim (hours)

FIG. 7B

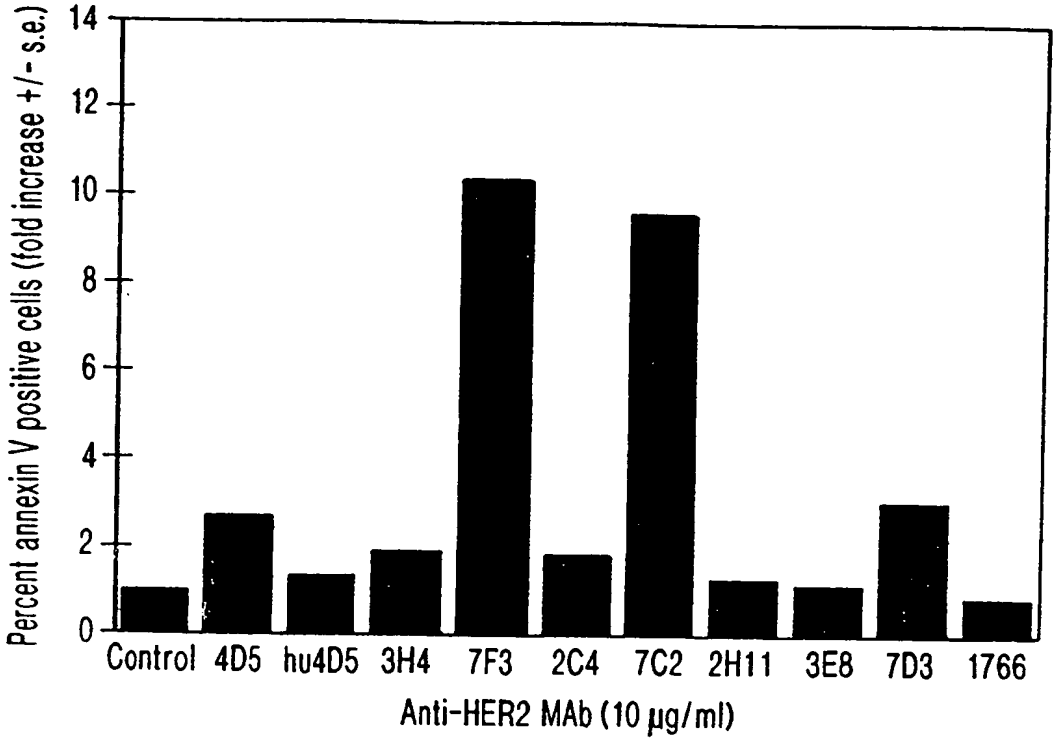

Percent annexin V positive cells (fold increase +/- s.e.)
14
12
10
8
6
4
2
0
Control
4D5
hu4D5
3H4
7F3
2C4
7C2
2H11
3E8
7D3
1766
Anti-HER2 MAb (10 µg/ml)

FIG. 8A

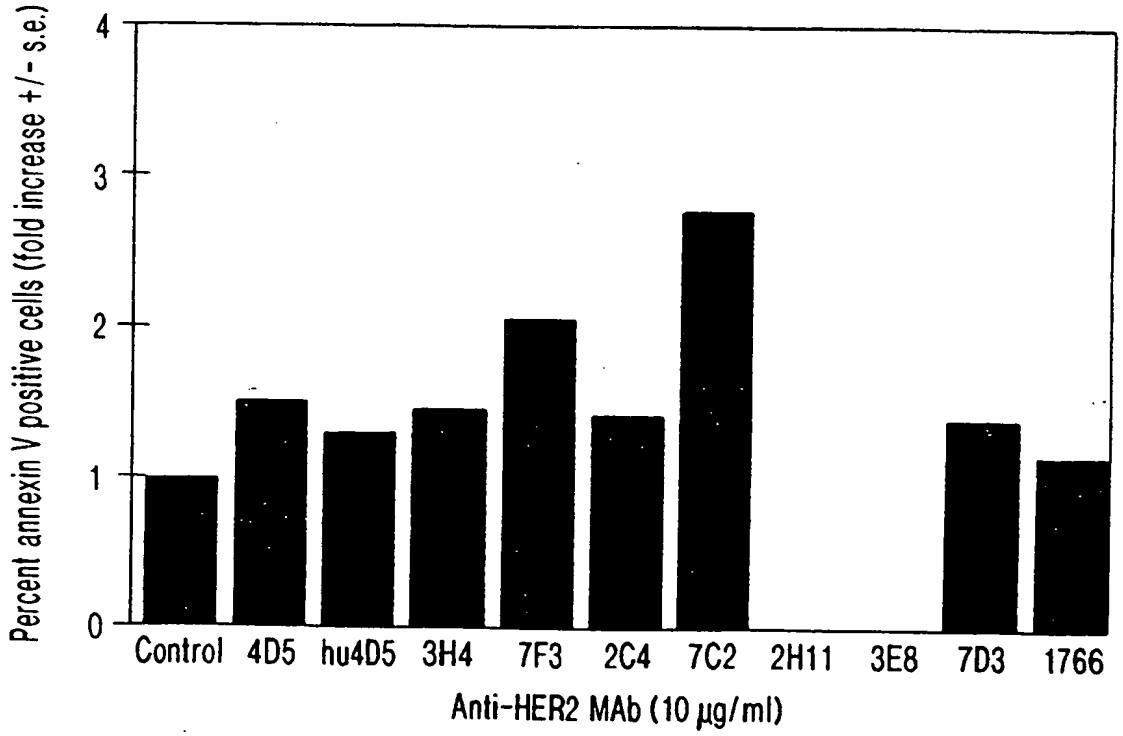

Percent annexin V positive cells (fold increase +/- s.e.)
4
3
2
1
0
Control
4D5
hu4D5
3H4
7F3
2C4
7C2
2H11
3E8
7D3
1766
Anti-HER2 MAb (10 µg/ml)

FIG. 8B

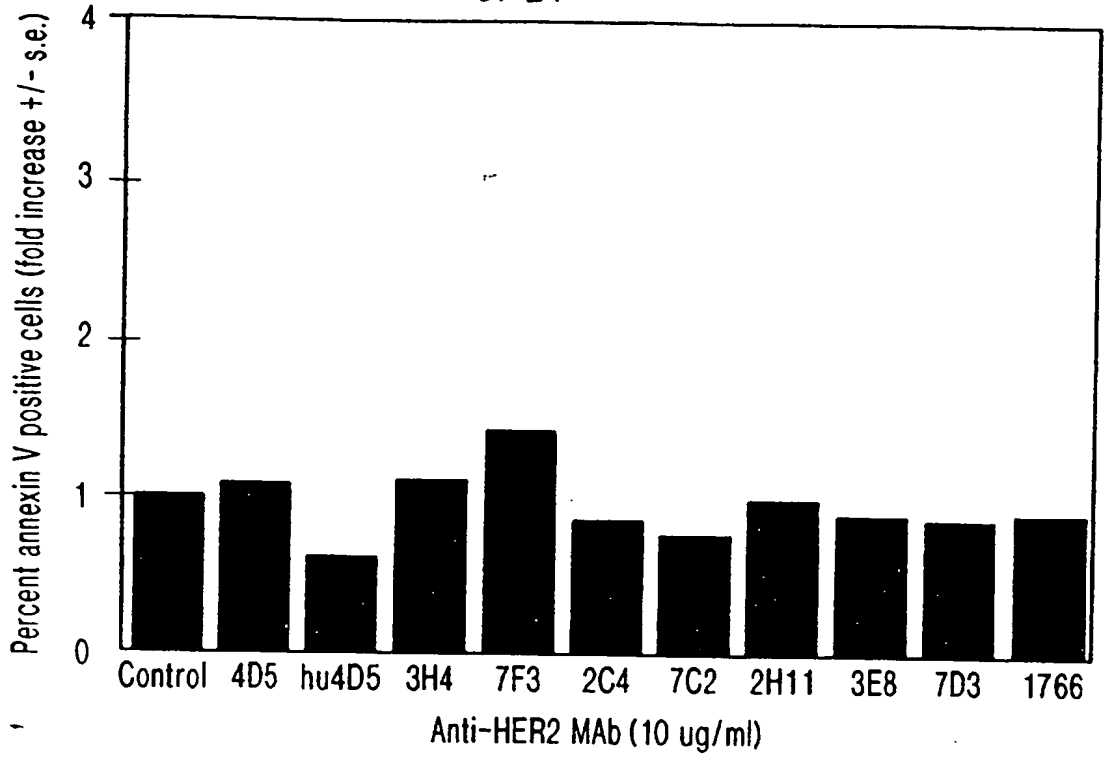

Percent annexin V positive cells (fold increase +/- s.e.)
0
1
2
3
4
Control
4D5
hu4D5
3H4
7F3
2C4
7C2
2H11
3E8
7D3
1766
Anti-HER2 MAb (10 ug/ml)

FIG. 8C

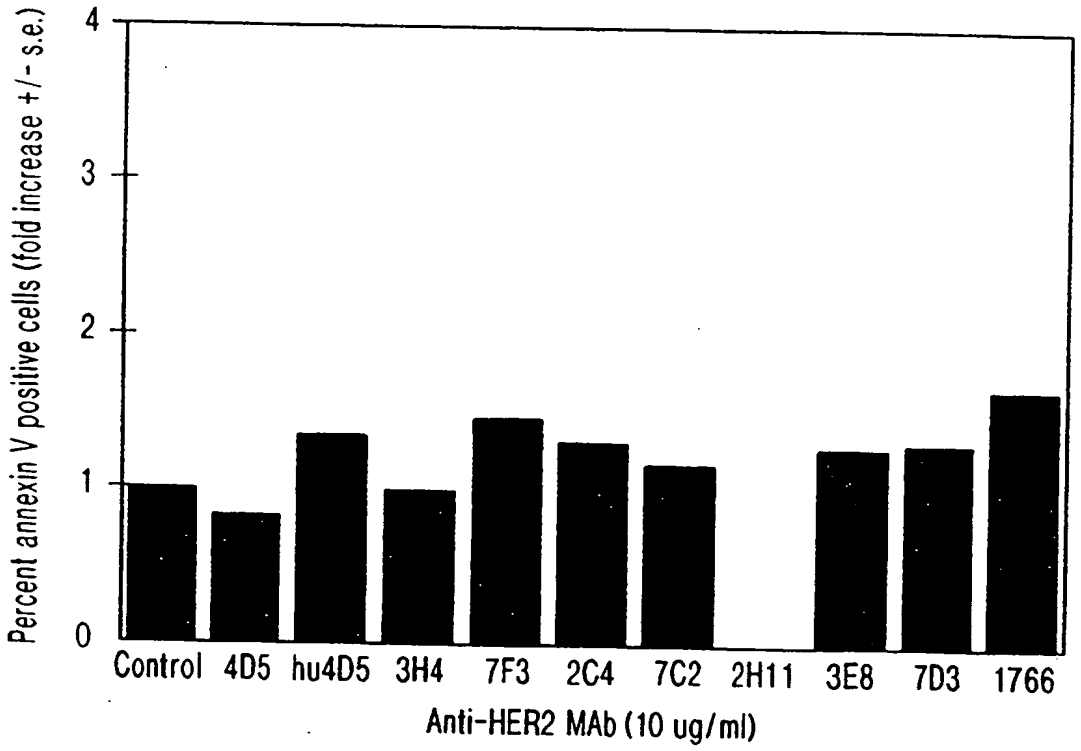

Percent annexin V positive cells (fold increase +/- s.e.)
0
1
2
3
4
Control
4D5
hu4D5
3H4
7F3
2C4
7C2
2H11
3E8
7D3
1766
Anti-HER2 MAb (10 ug/ml)

FIG. 8D

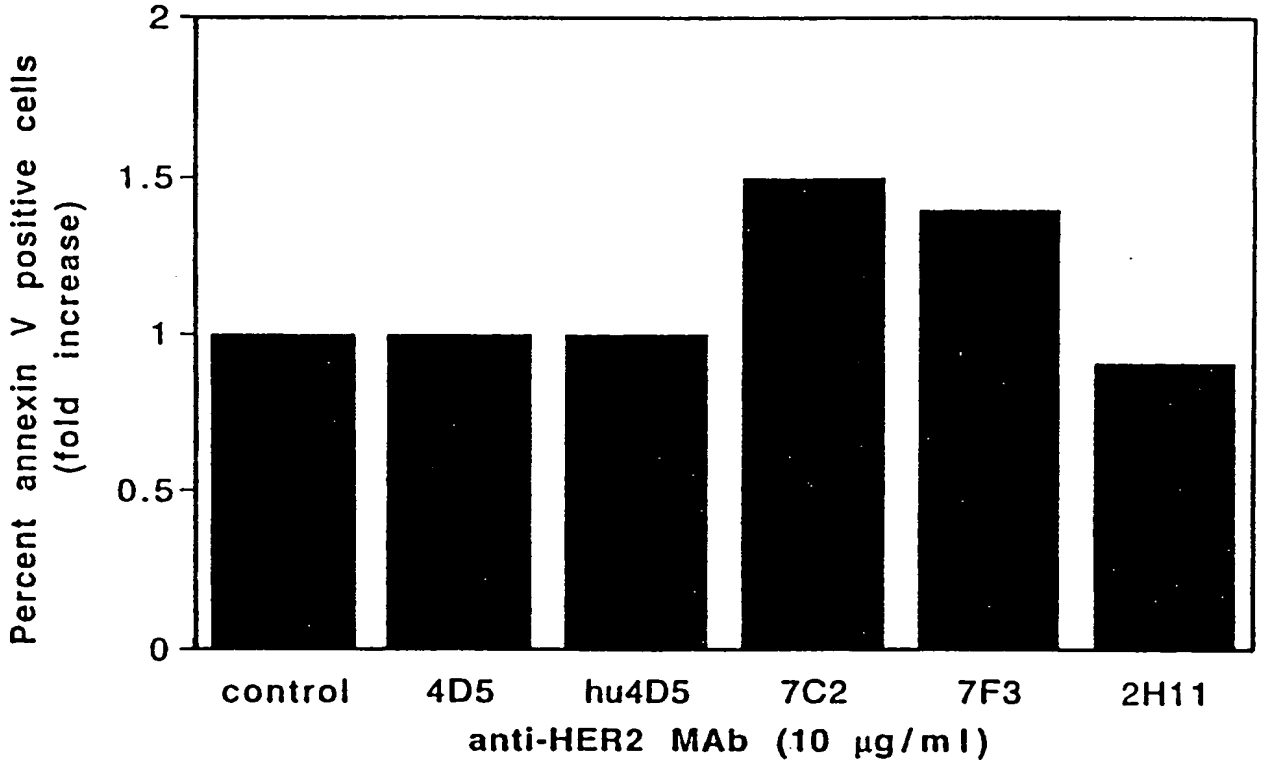

Percent annexin V positive cells
(fold increase)
2
1.5
1
0.5
0
control
4D5
hu4D5
7C2
7F3
2H11
anti-HER2 MAb (10 µg/ml)

FIG. 8E

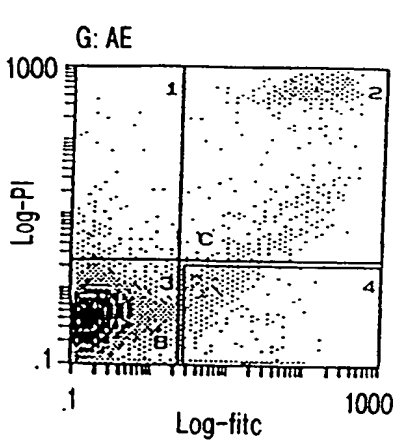



FIG. 9A

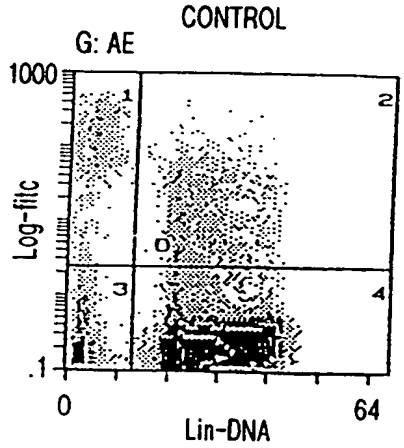



FIG. 9B

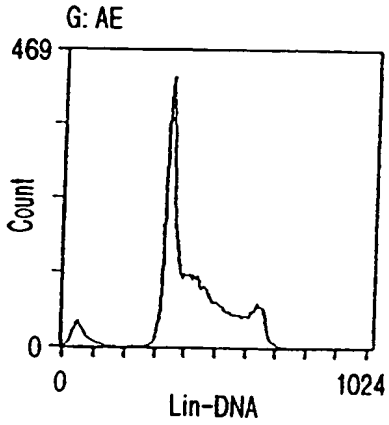



FIG. 9C

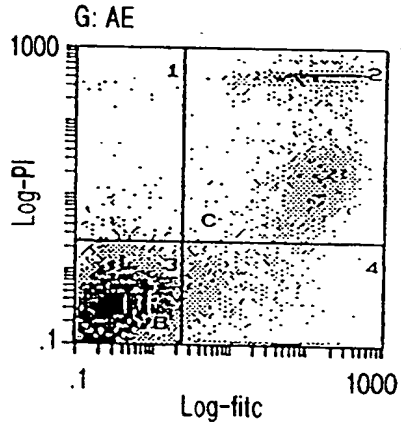



FIG. 9D

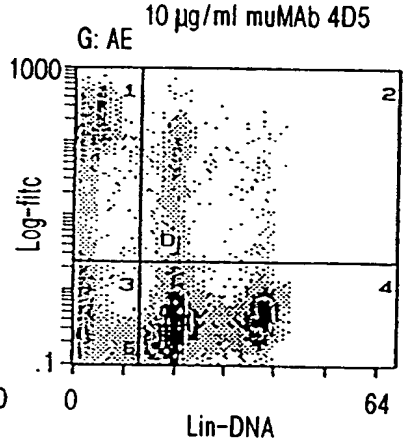



FIG. 9E

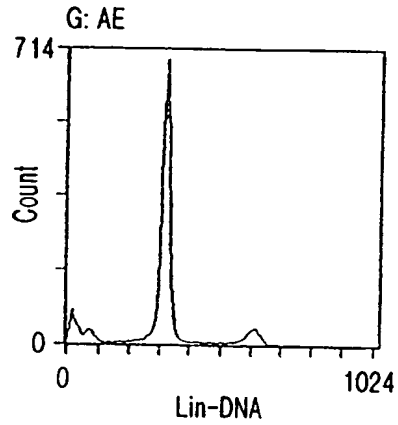



FIG. 9F

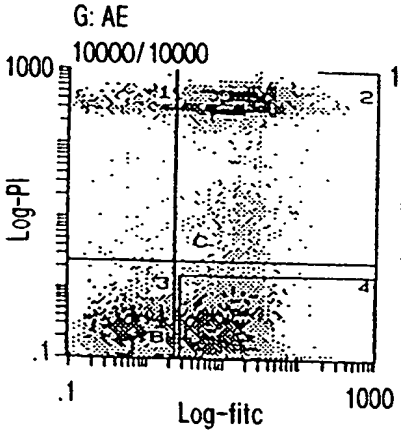



FIG. 9G

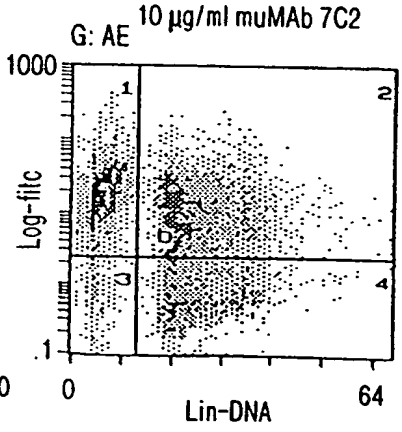



FIG. 9H

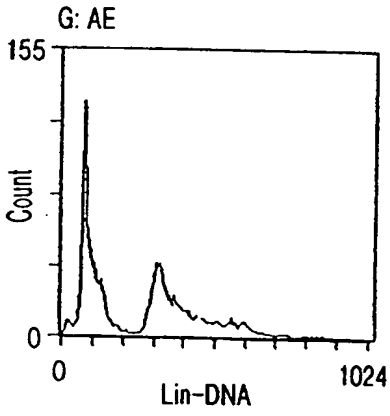



FIG. 9I

FIG. 10A

FIG. 10B

FIG. 10C

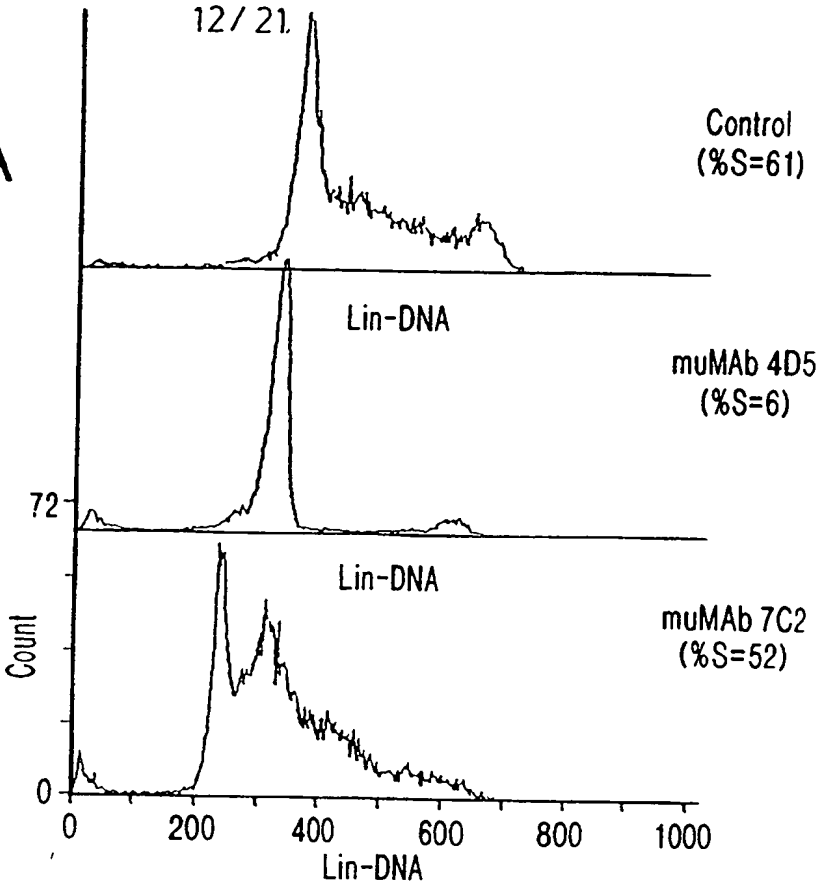
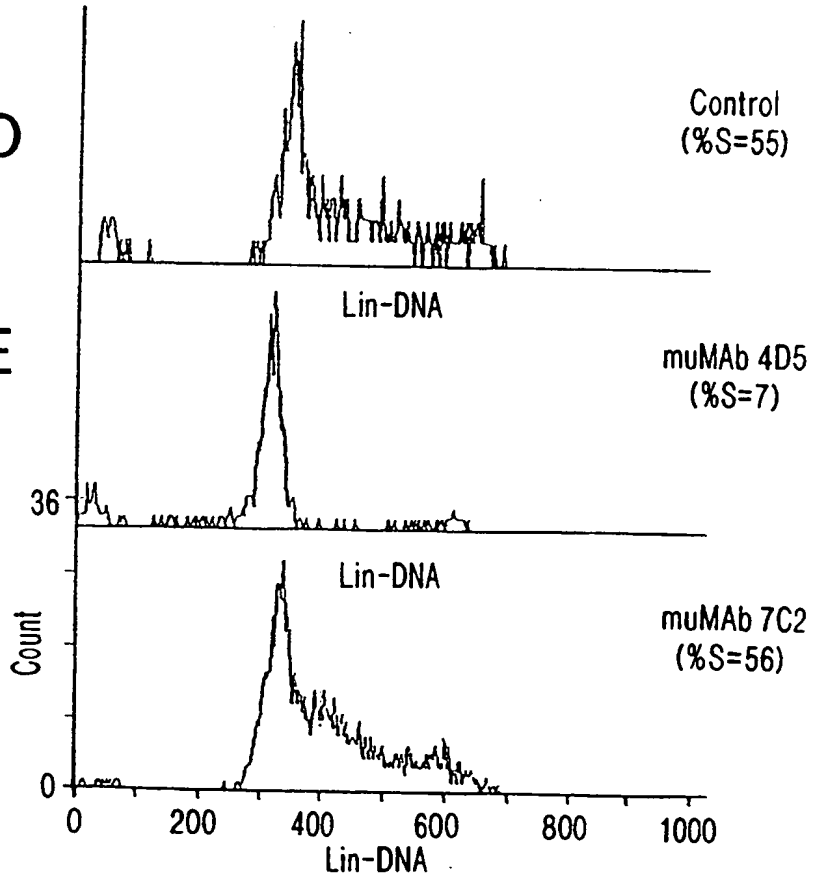



FIG. 10D

FIG. 10E

FIG. 10F



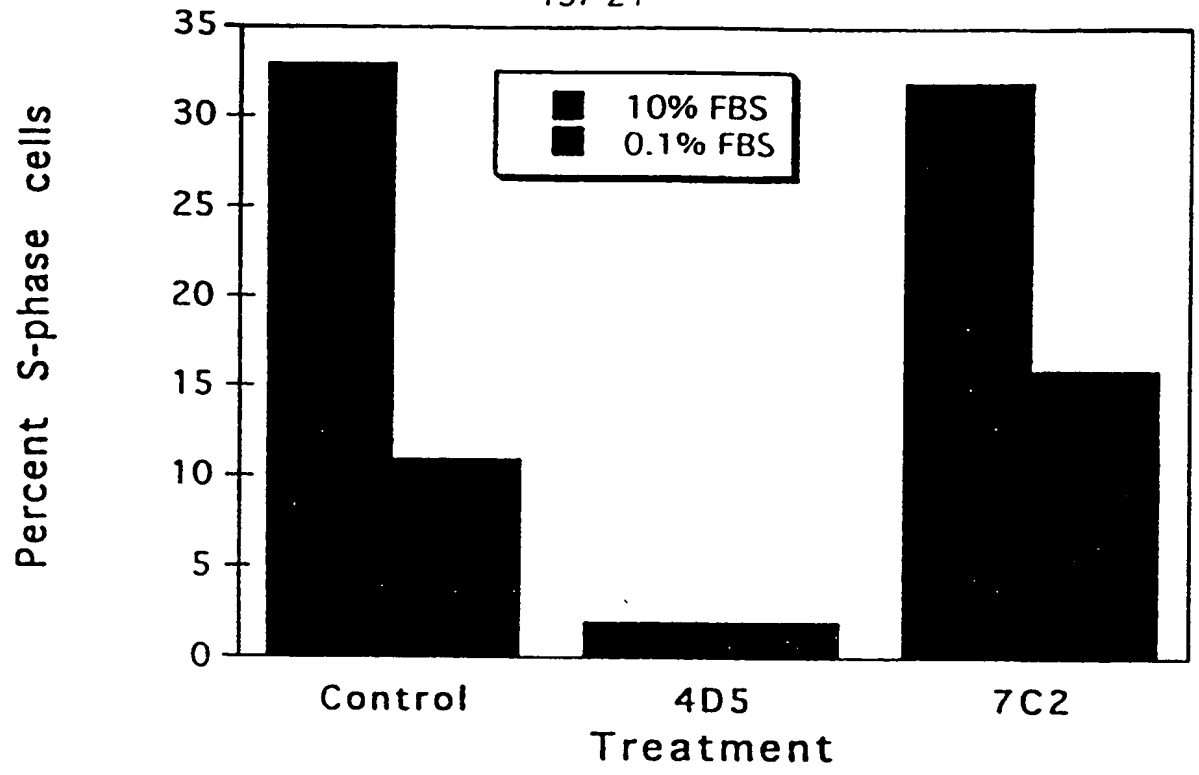



FIG. 11A

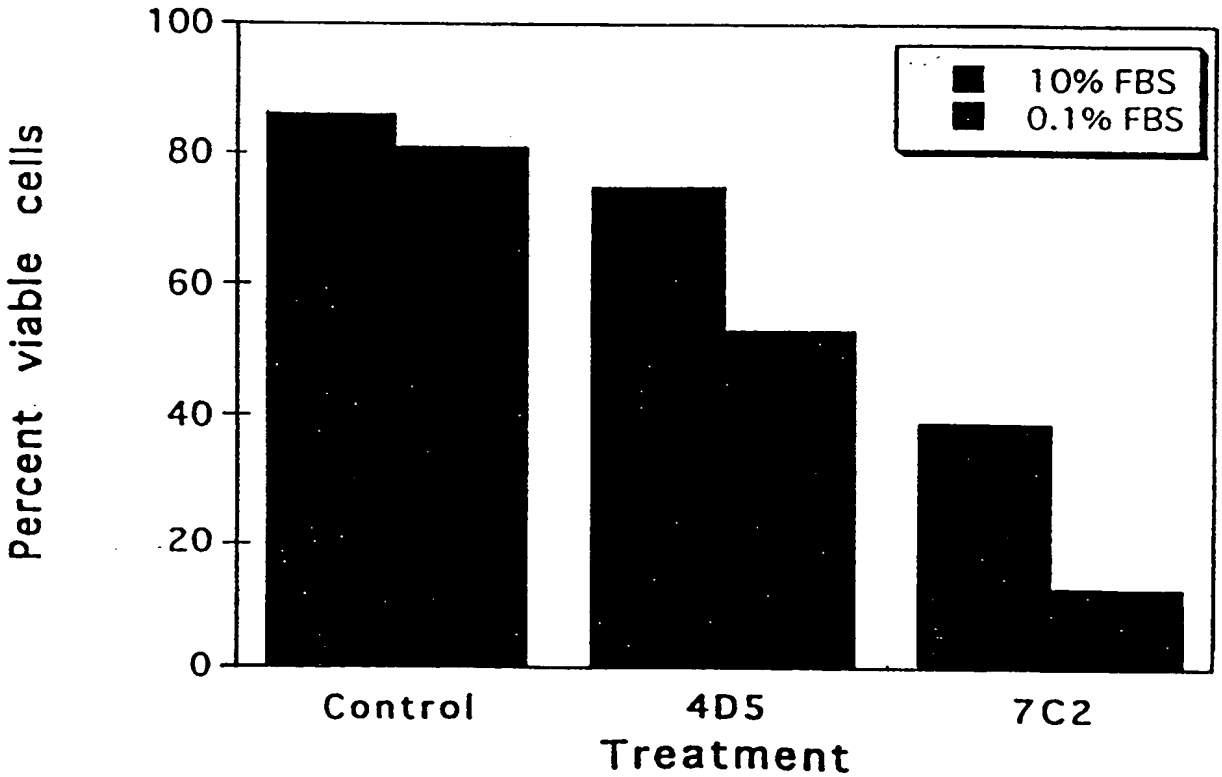



FIG. 11B

1 MELAALCRWGLLLALLPPGAA**STQV**CTGTDMKLRLPA
38 **SPETHLDMLRHLYQGC**QVVQGNLELTYLPTNASLSFL
75 QDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDN
112 YALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLTEI
149 LKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLID
186 TNRSRA

FIG. 12

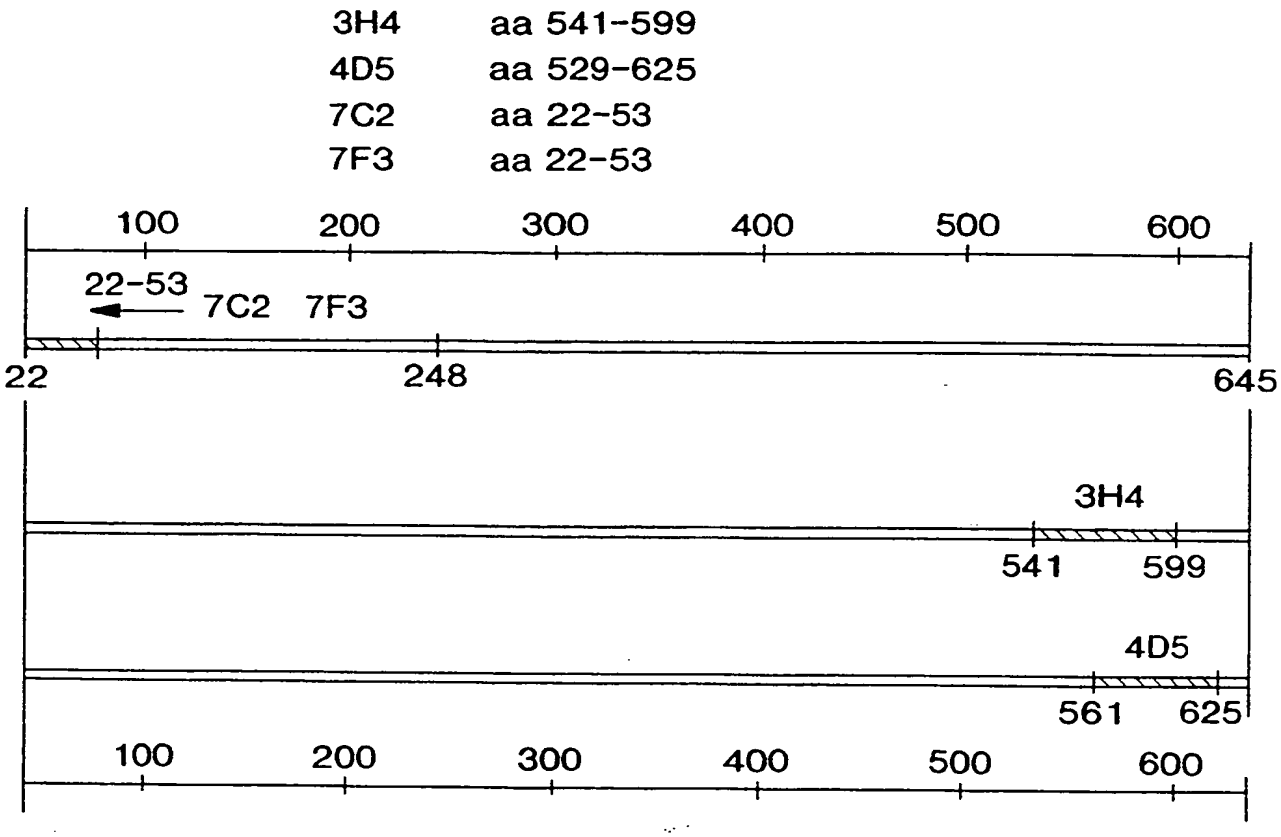

3H4 aa 541-599
4D5 aa 529-625
7C2 aa 22-53
7F3 aa 22-53
100
200
300
400
500
600
22-53
7C2
7F3
22
248
645
3H4
541
599
4D5
561
625
100
200
300
400
500
600

FIG. 13

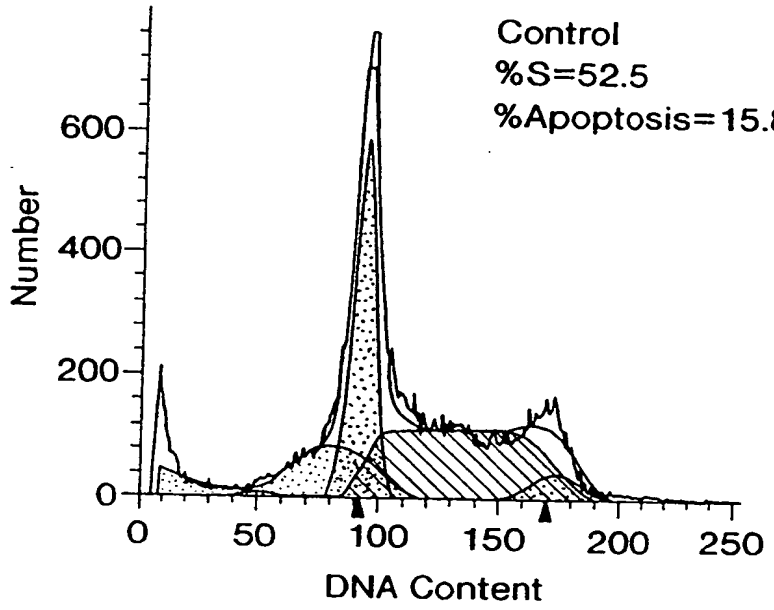



FIG. 14A

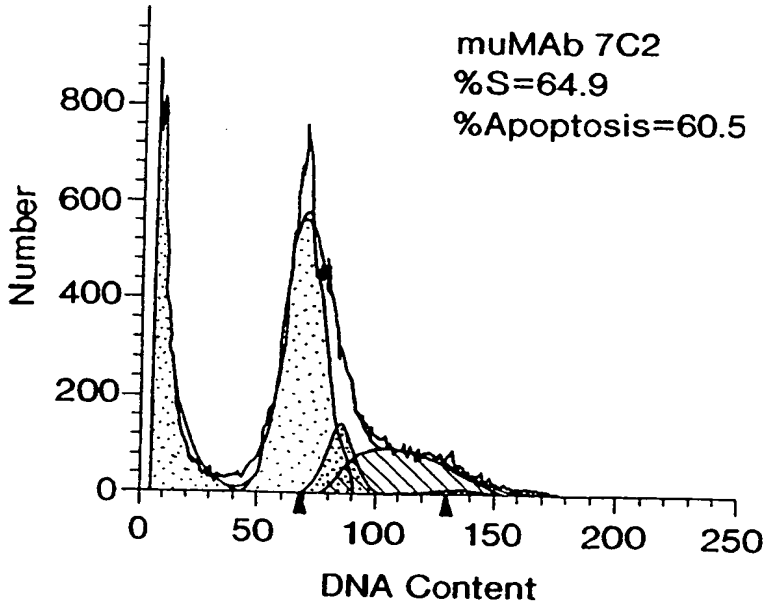



FIG. 14B

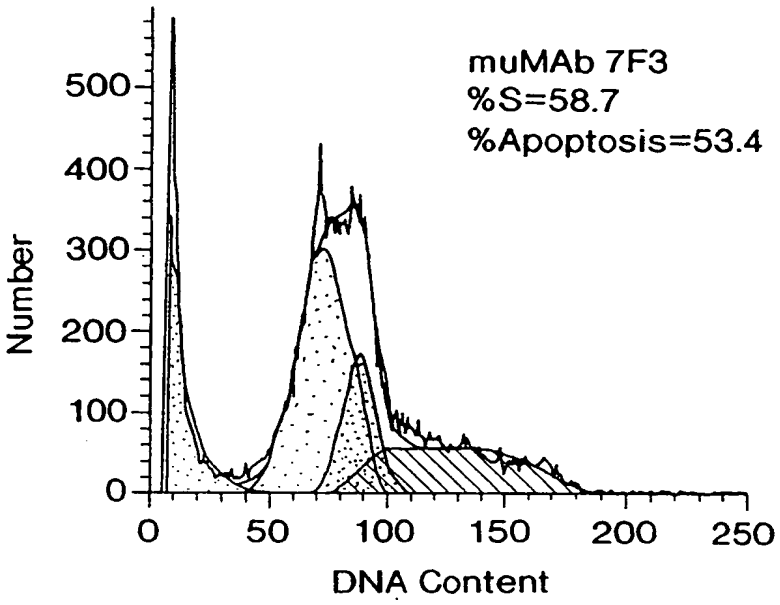



FIG. 14C

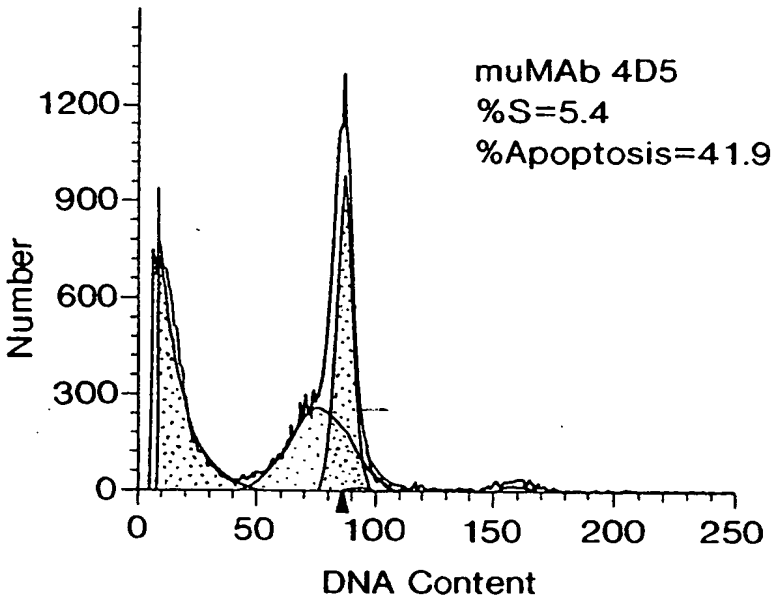



FIG. 14D

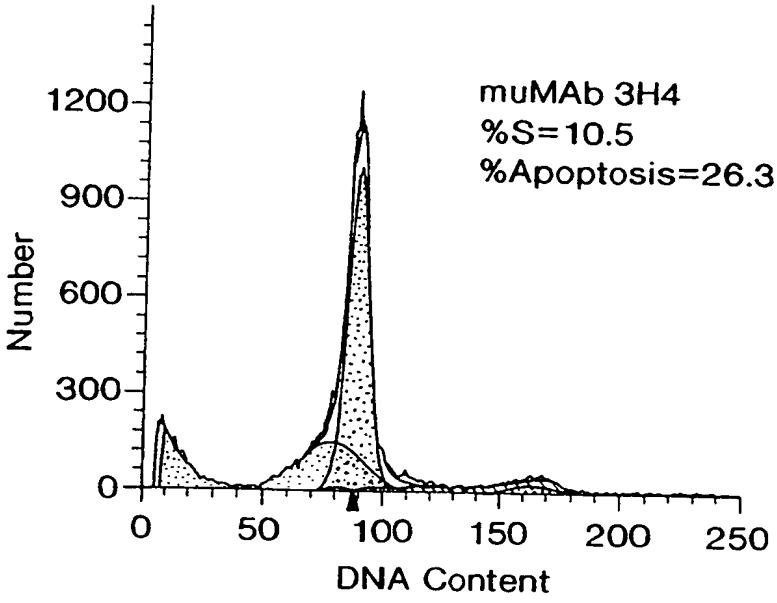

muMAb 3H4
%S=10.5
%Apoptosis=26.3
Number
1200
900
600
300
0
0
50
100
150
200
250
DNA Content

FIG. 14E

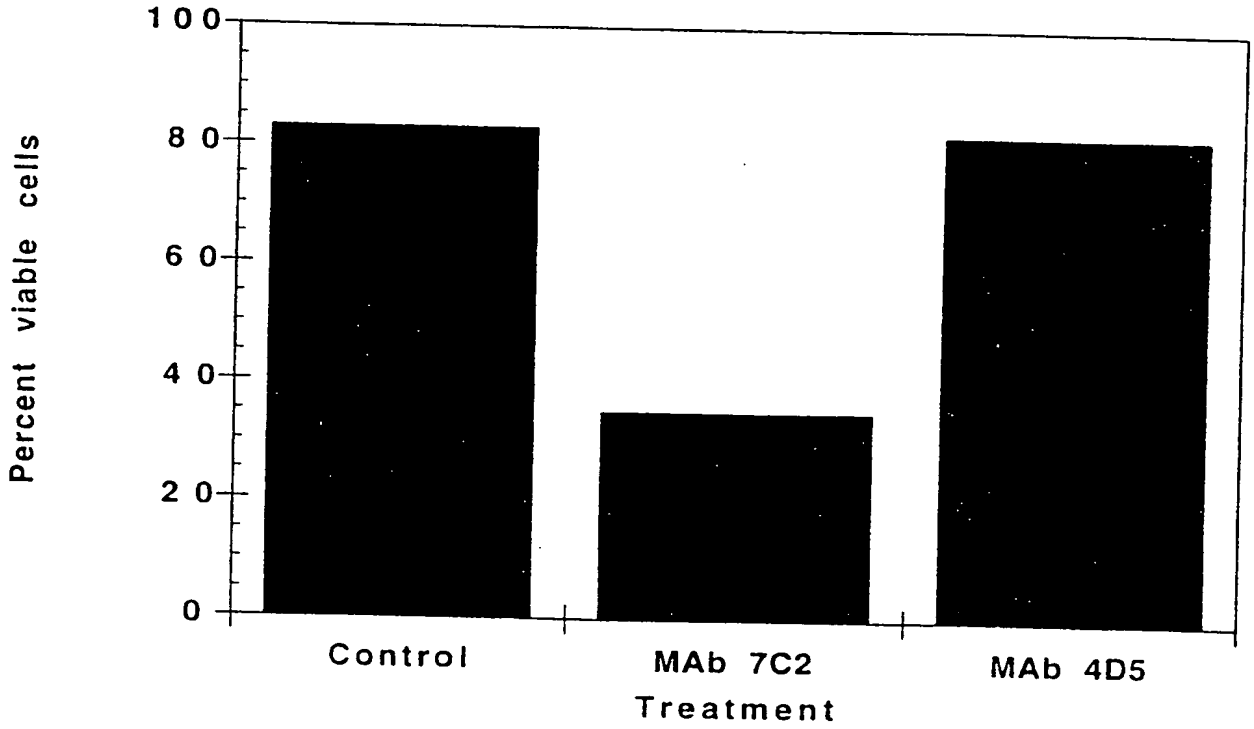

100
80
60
40
20
0
Percent viable cells
Control
MAb 7C2
MAb 4D5
Treatment

FIG. 15

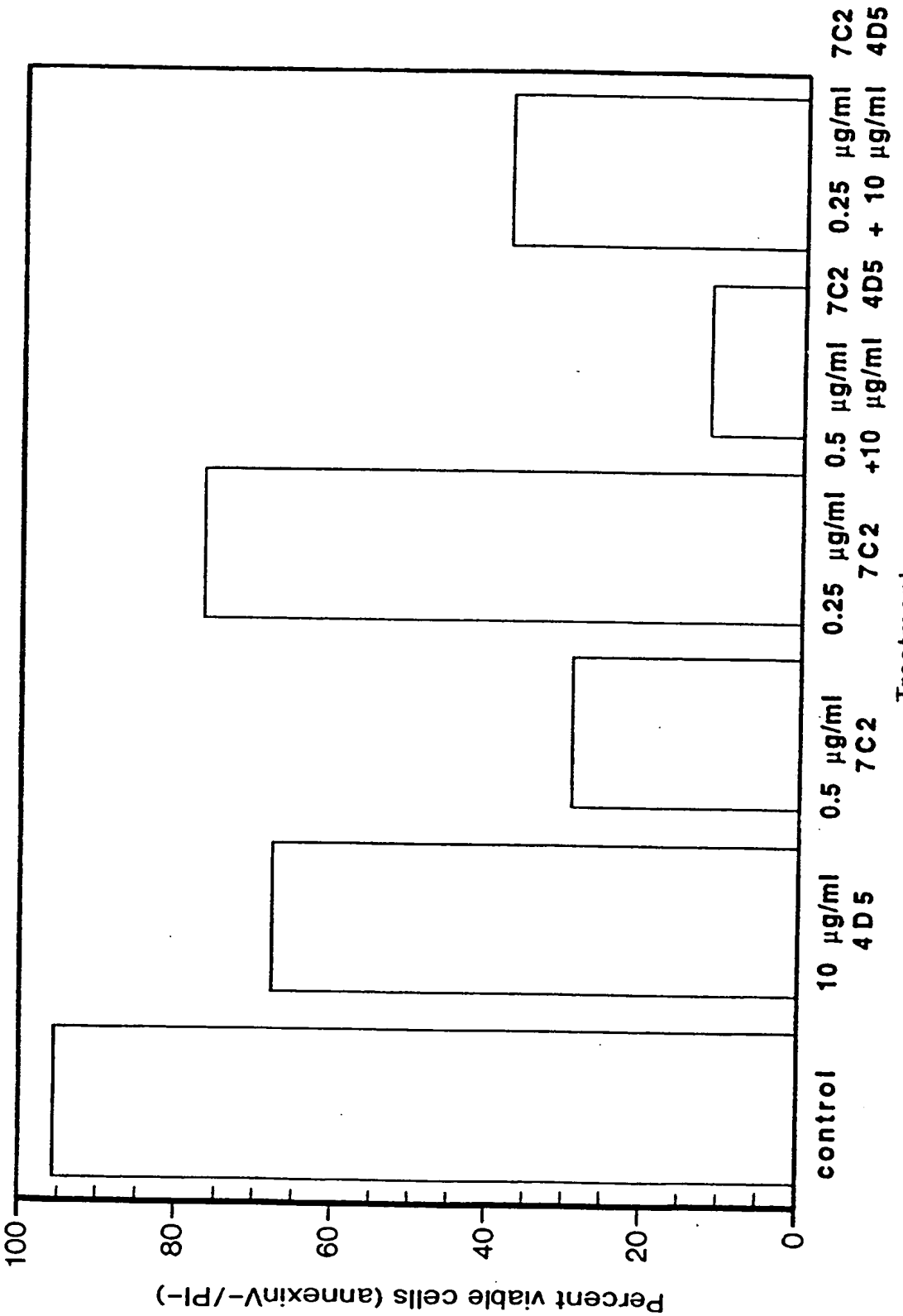

Percent viable cells (annexinV-/PI-)
100
80
60
40
20
0
control
10 µg/ml 4D5
0.5 µg/ml 7C2
0.25 µg/ml 7C2
0.5 µg/ml 7C2 +10 µg/ml 4D5
0.25 µg/ml 7C2 + 10 µg/ml 4D5
Treatment

FIG. 16

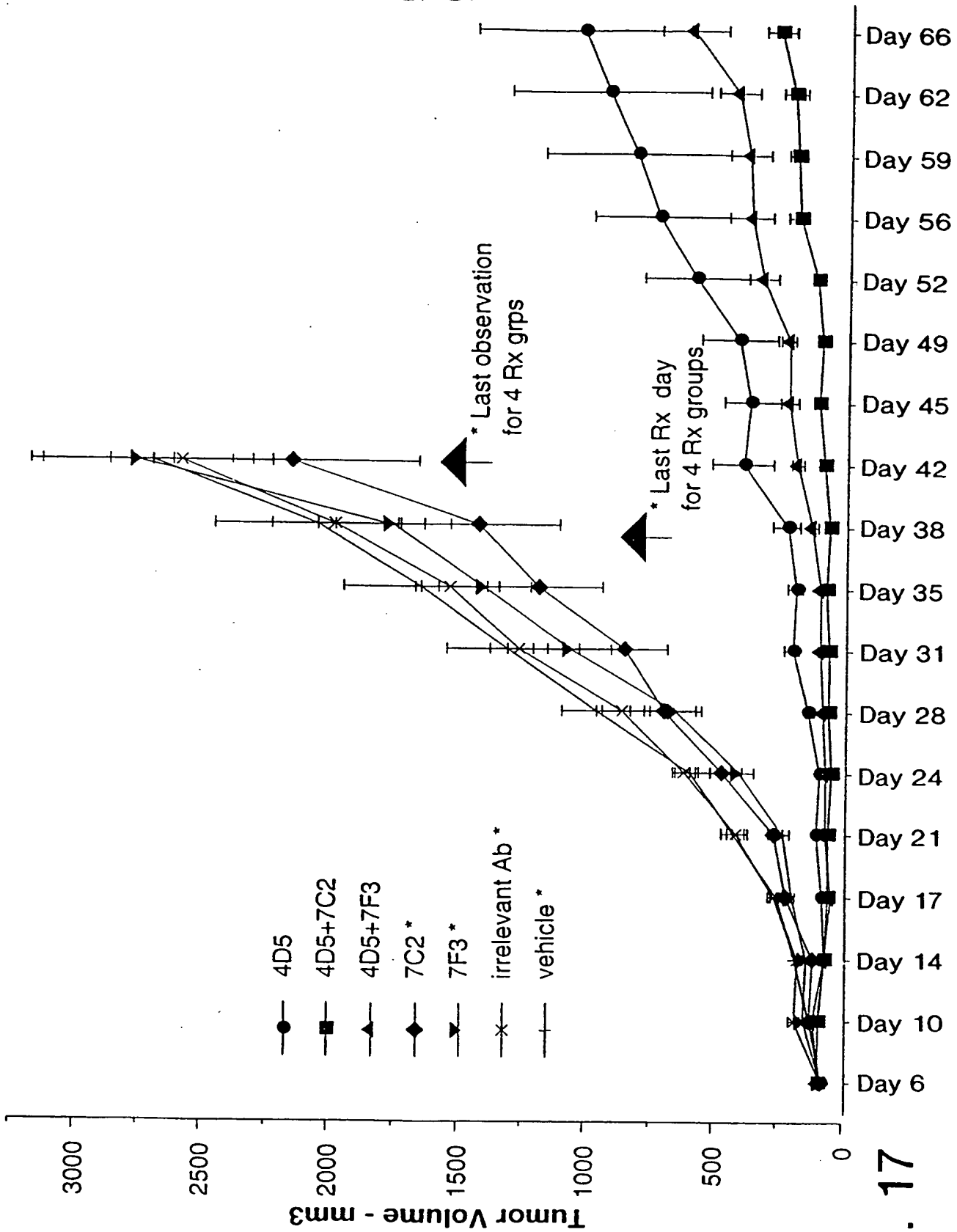

Tumor Volume - mm3
3000
2500
2000
1500
1000
500
0
Day 6
Day 10
Day 14
Day 17
Day 21
Day 24
Day 28
Day 31
Day 35
Day 38
Day 42
Day 45
Day 49
Day 52
Day 56
Day 59
Day 62
Day 66
* Last observation for 4 Rx grps
* Last Rx day for 4 Rx groups
4D5
4D5+7C2
4D5+7F3
7C2 *
7F3 *
irrelevant Ab *
vehicle *

FIG. 17

# INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 97/18385

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/13 C07K16/32 A61K39/395 C12N5/20 G01N33/577
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Y. KITA ET AL.: "ErbB receptor activation, cell morphology changes, and apoptosis induced by anti-Her2 monoclonal antibodies." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 226, no. 1, 4 September 1996, DULUTH, MN, USA, pages 59-69, XP002027856 see page 68, line 1 - line 16 see page 68, line 32 - line 36 --- -/-- | 1-5,9, 15, 20-23, 25-31, 40,41 |

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 February 1998 | 03. 03. 98 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Nooij, F |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | P. CARTER ET AL.: "Humanization of an anti-p185HER2 antibody for human cancer therapy." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, no. 10, 15 May 1992, WASHINGTON, DC, USA, pages 4285-4289, XP000275844 see abstract --- | 1,3,5,6, 18, 21-23, 25-31, 40,41 |
| X | K. DE SANTES ET AL.: "Radiolabeled antibody targeting of the HER-2/Neu oncoprotein." CANCER RESEARCH, vol. 52, no. 7, 1 April 1992, BALTIMORE, MD, USA, pages 1916-1923, XP002055534 see the whole document --- | 1,2,5,7, 21-27, 29-31 |
| X | G. DIGIESI ET AL.: "Production and characterization of murine mAbs to the extracellular domain of human Neu oncogene product GP185HER2." HYBRIDOMA, vol. 11, no. 4, August 1992, NEW YORK, NY, USA, pages 519-527, XP002028488 see abstract see table I ----- | 1,5, 21-23, 25-27, 29-31 |

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claim 26 (partially, as far as an vivo method is concerned) is directed to a diagnostic method practised on the human/animal body, and although claims 28-40 (partially, as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.